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Characterisation of a novel bacteriophage,  $\phi$ BHG1,  
and its interactions with its host *Rhodospseudomonas blautia*

by

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A thesis presented for the degree of  
Doctor of Philosophy

Department of Biological Sciences  
University of Warwick

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Declaration

The work contained in this thesis is the result of original research by myself under the supervision of Dr. G. S. Dow, unless otherwise stated. All sources of information have been acknowledged by means of reference. None of the work contained in this thesis has been submitted for any previous degree.

Hazel C. Gorham

November 1987

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### Abbreviations

BSA	bovine serum albumin
Ci	curie
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
ds	double stranded
EDTA	ethylenediaminetetra acetic acid
$\text{E} \cdot \text{m}^{-1} \cdot \text{s}^{-1}$	Einstein per meter per second
g	gravity
kb	kilobase
Ka	kanamycin
kPa	kilo pascal
M	molar
mM	millimolar
nm	nanometer
$\mu\text{m}$	micrometer
PAGE	polyacrylamide gel electrophoresis
RNA	ribonucleic acid
RNAase	ribonuclease
rpm	revolution per minute
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
TCA	trichloroacetic acid
TEMED	N,N,N,N'-tetramethylethylenediamine
Tris-HCl	tris (hydroxymethyl) amino methane
UV	ultraviolet
v/v	volume to volume
w/v	weight to volume

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## Summary

ØBHG1, a lytic phage specific for *Rhodospseudomonas blastica* has an icosahedral head of 62 nm diameter and a short, 39 nm long, non-contractile tail with a collar, base plate and short spikes. Caesium chloride density centrifugation of phage normally gave a single phage band corresponding to a density of  $1.3850 \text{ g cm}^{-3}$ , but occasionally a second band was observed ( $1.3838 \text{ g cm}^{-3}$ ). No apparent differences were observed between the two phage bands. The nucleic acid of ØBHG1 is double stranded DNA, with a G+C content of 50.6 mol %, and a molecular weight of 48 kb. The capsid proteins are observed ranging in molecular weight from 18,000 to 98,000 and corresponding to approximately 26% of the coding capacity of the genome. ØBHG1 adsorbed to both photosynthetically and chemoheterotrophically grown *Rp. blastica* at an identical rate of  $1.39 \text{ ml}^{-1} \text{ min}^{-1}$ . The phage has no specific cation requirement for adsorption. One-step growth curve studies of ØBHG1 with phage antisera gave a lag period of 80-100 min, a rise period of 100 min and a final burst size of  $25 \pm 2.5$  and  $30 \pm 2.1$  for chemoheterotrophically and photoheterotrophically grown cells respectively. Synchronisation of cells for one-step growth curves with potassium cyanide altered the burst size with photosynthetically grown cells. Adsorption of ØBHG1 was specific to the 'old' pole of *Rp. blastica*. Lysis of *Rp. blastica* by French pressing gave three distinct bands after differential sucrose density centrifugation corresponding to the cytoplasmic membrane and two cell wall fractions B1 and B2. Both cell wall fractions contained a dominant heat modifiable protein of molecular weight 35,000, and in addition cell wall fraction B1 contained two dominant proteins of Mr 14,500 and 15,000. Amino acid analysis suggests a difference in the amount of cross-linkage of the peptidoglycan between the two cell wall fractions. Comparisons of *Rp. blastica* with a spontaneous phage resistant mutant *Rp. blastica*<sup>19</sup> to which ØBHG1 fails to adsorb showed only trace amounts of the Mr 14,500 and 15,000 proteins in the resistant strain. Analysis of lipopolysaccharide from the two strains showed the loss of the neutral sugar 2-O-methyl-6-deoxyhexose and a reduction in the concentration of galactose in *Rp. blastica*<sup>19</sup>. Evidence suggests that an LPS/protein complex may function as the bacterial phage receptor and is required to inactivate ØBHG1 in vitro.

To my Family and Derek

## INTRODUCTION



### 1.1 Characteristics of the Rhodospirillaceae

The *Rhodospirillaceae* (purple non-sulphur bacteria) are a family of the suborder *Rhodospirillanae* (Pfennig, 1978). Member species are wide spread amongst soil and water environments, are capable of numerous growth modes (Pfennig, 1978) and are the most physiologically diverse and best studied group of anaerobic photosynthetic bacteria. Many of its members can grow under a wide variety of growth conditions demonstrating a high degree of metabolic flexibility. *Rhodobacter capsulatus* was found to be capable of five growth modes (Madigan and Gest, 1979): (i) anaerobic growth as a photoautotroph on  $H_2$  plus  $CO_2$  with light as the energy source; (ii) anaerobic growth as a photoheterotroph on various organic carbon sources with light as the energy source; (iii) growth as a fermentative anaerobe, in darkness, on sugars as sole carbon and energy sources; (iv) aerobic growth as a chemoheterotroph in the dark; and (v) aerobic growth as a chemoautotroph in the dark, with  $H_2$  as the source of electrons. The predominant growth mode of the *Rhodospirillaceae* however is that of anaerobic photoheterotrophy where organic compounds are assimilated into cell carbon using light as an energy source. Under these conditions, in most cases, the organic compounds also act as electron donors. The range of carbon sources that can be utilized is wide and includes, for example, fatty, organic and amino acids. The ability of members of the *Rhodospirillaceae* to grow in the alternative chemoheterotrophic mode of growth varies considerably within the family. In the presence of oxygen, energy is obtained solely from the endogenous chemical reactions within the cell, with oxygen as the terminal electron acceptor. As with photoheterotrophic growth, carbon is obtained from exogenous organic compounds.

Recently, Imhoff *et al.* (1984) proposed a rearrangement of the species and genera of the purple non-sulphur bacteria, based on recent taxonomic data. It has been proposed that the *Rhodospirillaceae* be composed of three genera *Rhodophila*, *Rhodobacter* and *Rhodopsseudomonas*. Bacteria of the genus *Rhodopsseudomonas* are generally found to be rod-shaped, exhibit budding, show asymmetrical division as a mode of reproduction, are motile by means of flagella and have intracytoplasmic membranes that are adjacent and parallel to the cytoplasmic membrane.

Eutrophic fresh water systems are a rich source of phototrophic bacteria, and in particular of those belonging to the genus *Rhodopsseudomonas* (Siefert *et al.*, 1978). *Rhodopsseudomonas blastica* was isolated from a small eutrophic freshwater pond (Eckersley and Dow, 1980). Whilst *Rp. blastica* has characteristics of the genus *Rhodopsseudomonas*, it has several distinct features. The cells are Gram-negative and rod shaped, 1 to 2.5  $\mu\text{m}$  long and 0.6 to 0.8  $\mu\text{m}$  wide. Some morphological variation is observed with some cells appearing more rod-shaped, similar to *Rhodobacter sphaeroides*. The developmental basis of such variants has so far not been investigated. In several *Rhodopsseudomonas* species motility has been reported to be lost with changes in cultural conditions, e.g. *Rhodopsseudomonas acidophila* and *Rhodopsseudomonas palustris* (Tauschel and Hoeniger, 1974). However, *Rp. blastica* was found to be non-motile under all conditions tested. Growth is by sessile polar budding, i.e. no tube or filament is formed between the mother and daughter cell and in this respect it resembles *Rp. acidophila* (Pfennig, 1969) and *Rp. sulfoviridis* (Keppen and Gorlenka, 1975). Division of *Rp. blastica* produces two identical cells indicating division is symmetrical. However, evidence that the cell cycle of *Rp. blastica* is in fact dimorphic has come from slide culture studies.

After division the 'daughter' cell does not divide until a further round of division has been accomplished by the mother cell. This suggests that, before it is reproductively competent, the daughter cell must pass through a phase of maturation (Eckersley and Dow, 1980).

Few other studies have been undertaken with *Rp. blasticus*. Sani (1985) studied the enzyme ribulose biphosphate carboxylase oxygenase, Zannoni (1984), the spectral and functional characterisation of membrane fragments, and Tegtmeyer *et al.* (1985) the characteristics of its lipopolysaccharide.

## 1.2 The Structure of the Cell Envelope in the Rhodospirillaceae

Ultrastructurally, all photosynthetic eubacteria are Gram-negative (Pfennig and Trüper, 1974). The walls of Gram-negative bacteria, when studied by electron microscopy, contain a dense uniform inner layer, only 2-3 nm wide (the so called rigid or R-layer) which is overlain by a somewhat thicker outer layer of 8-10 nm. The fine structure of this outermost layer is virtually indistinguishable from that of a unit membrane and is therefore often referred to as the outer membrane (Weckesser *et al.*, 1972; Drews *et al.*, 1978). Figure 1a shows a general model for the cell envelope of a Gram-negative bacterium. The cell envelope is a complex structure and consists of three layers (Tipper and Wright, 1979).

### 1.2.1 Cytoplasmic membrane

The inner, cell or cytoplasmic membrane is a phospholipid bilayer (- unit membrane) with associated proteins forming a fluid mosaic layer

Figure 1a

General model for the cell wall of a Gram-negative bacterium

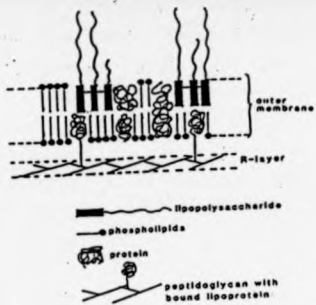
(adapted from Wilkinson, 1977).

Figure 1b

A schematic representation of the organisation of the intact peptidoglycan sac. NAG and NAM designate residues of N-acetylglucosamine and N-acetylmuramic acid respectively

Figure 1c

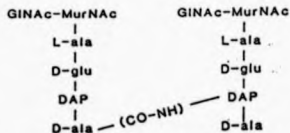
Representation of the mode of cross-linking between the terminal carboxyl group of D-alanine on one subunit of NAM and the free amino group of DAP (diaminopimelic acid) on an adjacent NAM subunit



a



b



c

(Singer and Nicholson, 1972). Although the width of the unit membrane is fixed, its surface area is not. Morphological and fine structure analysis of photosynthetically grown *Rs. rubrum* revealed vesicular membrane invaginations that were absent from aerobic, chemoheterotrophically grown cells. These were proposed as the location of the photosynthetic apparatus and were designated the intracytoplasmic membrane (ICM) (Vatter and Wolfe, 1958). Within the *Rhodospirillaceae*, a variety of types of arrangements of ICM are known (Kelly and Dow, 1985).

A variety of independent experimental approaches have shown that the cytoplasmic membrane is continuous with the ICM. These included electron microscopy (Golacki and Gelze, 1975), the isolation of bacteriochlorophyll *a* depleted areas of the membrane system in phototrophically grown cells, and the identification of putative "invagination sites" of the cytoplasmic membrane on sucrose gradients (Inamine *et al.*, 1984).

The bacterial cytoplasmic membrane is an important centre for metabolic activity. It contains many different proteins, accounting for approximately 50-60% of the dry weight of the cytoplasmic membrane, each of which probably has a specific catalytic function. The major classes of proteins include permeases and biosynthetic enzymes. In addition, the cytoplasmic membrane contains important components of the ATP generation system. Considerable work has been carried out on the protein components in the ICM of three purple non-sulphur bacteria *Rs. rubrum*, *Rb. sphaeroides*, and *Rb. capsulatus*. Several enzyme activities have been demonstrated in the ICM preparations from these species, including succinate dehydrogenase, NADH oxidase and ATPase activity

(Neiderman and Gibson, 1971; Thore et al., 1969; Reed and Raveed, 1972). These enzyme activities are often used as "markers" for the membrane during purification.

Phospholipids are the major lipids of the bacterial cytoplasmic membrane and account for about 20-30 percent of their dry weight. In the case of *Rb. sphaeroides* the major phospholipids are phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylcholine (PC) (Gorcheim, 1968). However, as a group, the *Rhodospirillaceae* have a very heterogeneous lipid composition (Imhoff et al., 1982). Many species contain large amounts of ornithine-containing lipids, especially *Rm. vannielii* (Imhoff et al., 1982). Oniski and Neiderman (1982) studied the effect of cultural conditions on the phospholipid composition of *Rb. sphaeroides*. Results indicated that there was no apparent differences between photoheterotrophically or chemoheterotrophically grown cells.

#### 1.2.2 Peptidoglycan

Peptidoglycan (PG) is a closely related family of polymeric molecules that constitute approximately 1% of the cell wall of Gram negative bacteria compared to 40-90% in Gram-positive bacteria. The monomeric constituents of PG are two acetylated amino sugars, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), and a small number of amino acids some of which (e.g. diaminopimelic acid (DAP)) are never found in cellular proteins. In one dimension the two acetylated amino sugars form glycan strands composed of alternating residues of NAG and NAM in  $\beta$  1-4 linkage. The second dimension of peptidoglycan is formed with parallel glycan chains cross-linked by peptide bridges (Fig. 1b). The NAM units are substituted on the acetyl group by tetrapeptides which are in turn cross-linked by an interpeptide bridge (Fig. 1c). The most

common sequence of tetrapeptide is NAM - L-alanine - D-glutamic acid - L-diaminopimelic acid - D-alanine. The covalent bonding results in a net-like structure. The predominant role of peptidoglycan in the bacterial cell wall is to provide strength and rigidity.

Cell wall fractions can be identified by the presence of two compounds unique to peptidoglycan, diaminopimelic acid (DAP) and muramic acid (Mur), (an essential component of NAM), peptidoglycan being absent in any membrane structure such as the cytoplasmic membrane. These two compounds can be detected by amino acid analysis where amino compounds are measured in a colourimetric assay involving the dye ninhydrin. Ideally, analysis should result in a ratio of Mur:DAP of one, since in peptidoglycan each NAM unit has a tetrapeptide chain associated with it, containing DAP. However, there are cases where the structure of the peptidoglycan is different to that described above. Schemelzer *et al.* (1982) reported that for the peptidoglycan from *Rp. viridis*, at least 70% of the glucosamine residues in the glycan chain lack N-acetyl moieties. Evidence has also been provided for a similar structure in *Rp. sulfoviridis* and *Rp. palustris*. Jørgens *et al.* (in press) determined that approximately 10-30% of glucosamine residues in *Rm. vannielii* were not N-acetylated and that peptidoglycan was only 60% cross-linked.

### 1.2.3 Outer membrane

External to the thin peptidoglycan layer of the bacterium is the outer membrane (OM). Although it has the width and fine structure of a typical unit membrane, it differs in its chemical composition from that of the cytoplasmic membrane. Its major constituents are proteins, phospholipids and lipopolysaccharide (LPS). The phospholipids are



structurally similar to those of the cytoplasmic membrane, but the proteins are largely, if not entirely, different. Separation of the inner and outer membranes can be achieved by either French pressing cells and removing cell debris, or mechanically disrupting sphaeroplasts prepared by lysozyme-EDTA treatment, followed by density gradient centrifugation. Only in the latter treatment is the peptidoglycan removed and thus many studies are on 'purified' cell walls containing outer membrane and peptidoglycan. Studies with *Rs. rubrum* (Oelze *et al.*, 1975; Collins and Neiderman, 1976a) and *Rb. sphaeroides* (Guillotin and Reiss-Husson, 1975; Ding and Kaplan, 1976) have all been carried out on chemoheterotrophically grown cells. Chemoheterotrophic growth has generally been chosen in preference to photosynthetic growth, in an attempt to reduce contamination by cytoplasmic membrane since as previously described, photosynthetic growth results in the formation of intracytoplasmic membranes. However, care must be taken to ensure that oxygen does not become limiting under chemoheterotrophic conditions since oxygen starvation is also a stimulus for intracytoplasmic membrane synthesis.

The protein profile of the outer membrane fraction from *Rs. rubrum* upon denaturing gel electrophoresis shows relatively few, but highly abundant species. This contrasted with that from the cytoplasmic membrane, which is more complex. The protein composition of the *Rb. sphaeroides* outer membrane has been characterised in great detail (Baumgardner *et al.*, 1980). It was found that a major protein of Mr 47,000 was heat modifiable. Below 75°C the protein formed three variant hetero-oligomers of Mr 68,000, 72,000 and 75,000 with polypeptides of Mr 21,500, 26,500 and 27,000 respectively. At 75°C or above the aggregates disassociate to the Mr 47,000 subunit and the lower Mr polypeptides.

Further immunological studies by Deal and Kaplan (1983a) supported the above observations and later identified the polypeptides as proteolipids (Deal and Kaplan, 1983b). Weckesser *et al.* (1984) isolated this polypeptide from saline extracts of whole cells and found that it exhibited strong porin activity. They found that the porin existed as a homo-oligomer of Mr 68,000 which dissociated upon EDTA or heat treatment to the Mr 47,000 form. Such disparities are typical for porins from Gram-negative bacteria (Nikaido and Vaara, 1985).

The Mr 21,500 outer membrane polypeptide from *Rb. sphaeroides* was thought to be associated with the peptidoglycan because of its altered electrophoretic mobility after lysozyme digestion. Also, an Mr 8,000-10,000 lipoprotein may be equivalent to the Braun lipoprotein of *Escherichia coli* (Baumgardner *et al.*, 1980; Braun and Rahn, 1969).

Flammann and Weckesser (1984a) characterised the outer membrane from *Rb. capsulatus*. The protein profile was found to be dominated by a single heat modifiable polypeptide of Mr 69,000. When solubilised at temperatures above 70°C, a polypeptide of Mr 33,000 was observed. Saline extracts of cells enriched for this protein showed porin activity *in vitro* (Flammann and Weckesser, 1984b).

#### 1.2.4 Lipopolysaccharide

The other major components of the bacterial outer membrane lipopolysaccharides (LPS). These are complex molecules with molecular weights above 10,000 and varying widely in their chemical composition both within and between groups of Gram-negative bacteria. The classic model for bacterial LPS was proposed by Luderitz *et al.* (1971) for the products from smooth ("wild-type") strains of *Salmonella*. As shown in

Figure 2, the end of the molecule distal from the cell surface contains a hydrophilic oligo-saccharide region of three to four sugars repeated many times. This portion of the LPS is the most variable, accounting for the numerous serological types that are present even within a given species. The oligosaccharide is often referred to as the "O-antigen" in serological typing. Most Gram-negative bacteria are "smooth", their cell surfaces bearing O side chains, but "rough" mutants, in which the LPS has lost its O side chains, may arise.

Adjacent to the oligosaccharide chain is a less variable zone formed from a heterogeneous mix of hydrophilic molecules. This intermediate region of the LPS, called the "R-core" is divided arbitrarily into the inner and outer core regions. The outer core region, closest to the O-antigen, is composed of hexoses and N-acetylated hexosamines. The inner core region contains two types of compounds unique to bacteria, 2-keto-3-deoxyoctonic acid (KDO) and glyceromannohaptose (Hep). The former is often used as a "marker" for the identification of outer membranes (Omata and Murata, 1983).

The remainder of the LPS constitutes the hydrophobic end, termed the "lipid A". The chemical back-bone of the structure is a glucosamine disaccharide to which fatty acids are attached through either amide or ester linkages. The aliphatic side chains of the fatty acids are saturated. Lipid is attached to the R core by glycosidic linkage between the 3'-hydroxyl group of glucosamine units and KDO (Hase and Rietschel, 1976).

The function of the LPS is probably to endow the cell surface with hydrophilicity, while at the same time maintaining an effective

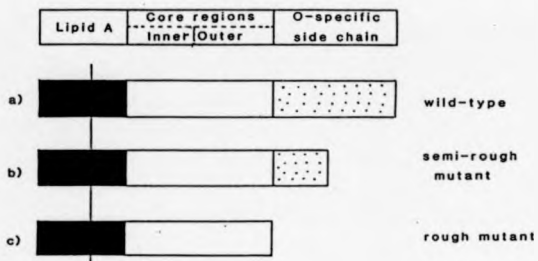


Figure 2

The lipopolysaccharide of the Gram-negative bacterium *Salmonella typhimurium*. Three regions are defined: lipid A, a core oligosaccharide with inner (proximal to the lipid) and outer regions, and the polysaccharide O side chain. a) wild-type; b) semi-rough mutant; c) rough mutant.

permeability barrier (Nikaido and Varra, 1985). The composition of LPS from photosynthetic bacteria and especially the *Rhodospirillaceae*, has been extensively investigated and has been reviewed by Weckesser *et al.* (1979). The lipid A moieties from the member species show different toxicities (Galanos *et al.*, 1977) and species specificity. This combined with studies on the sugar residues from the O-antigen side chains, has been useful in taxonomic studies of the *Rhodospirillaceae* (Draws *et al.*, 1978; Weckesser *et al.*, 1979). Such studies have indicated close relationships between *Rhodopseudomonas palustris*, *Rhodopseudomonas viridis* and *Rhodopseudomonas sulfoviridis* (Ahamed *et al.*, 1982) and between *Rhodocyclus gelatinosus* and *Rhodocyclus tenuis* (Weckesser *et al.*, 1977). These, to some extent agree with phylogenetic studies by Imhoff *et al.* (1984). Tegtmeyer *et al.* (1985) characterised the LPS of *Rp. blastica* and found a unique neutral sugar 2-O-methyl-6-deoxyhexose.

Cultural conditions greatly affect the structure of LPS. Studies on *Salmonella anatum* showed that after growth at 20°C, LPS contained more unsubstituted core molecules than the product from cells grown at 30-35°C (McConnell and Wright, 1979). The LPS from a *Pseudomonas aeruginosa* strain grown at 19°C was defective in phage-binding due to the absence of fucosamine from side chains (Cadieux *et al.*, 1983). Most studies have used LPS isolated from batch cultures grown to stationary phase. However, it is important to realise that the age of a culture as well as cultural conditions can effect the structure of LPS. When Hrabrak *et al.* (1981) examined *Rhizobium trifolii* LPS by changes in its immunological determinants they found that the relative quantity of several monosaccharide components increased with culture age.

### 1.2.5 Lipoproteins

As described above, the outer membrane and peptidoglycan layer are very complex and chemically distinct from one another. However, they physically interact such that the outer membrane is not completely independent of the layer beneath it.

In *E. coli*, peptidoglycan is covalently linked to a lipoprotein, Brauns lipoprotein (BLP) via a trypsin sensitive bond between diaminopimelic acid and lysine (Braun and Rehn, 1969). The hydrophobic portion of the protein reaches up into the hydrophobic fluid bilayer of the outer membrane (Fig. 1a). BLP is one of the most abundant membrane proteins and is present in two forms, one-third as the bound form covalently linked to the peptidoglycan and the remainder as the free form (Hirashima *et al.*, 1973). Numerically (Di Rienzo *et al.*, 1978), every tenth to twelfth DAP residue has lipoprotein attached. Ichihara *et al* (1981) characterised a new lipoprotein which although not covalently bound, is believed to be closely associated with the peptidoglycan layer. Baumgardner *et al.* (1980) observed in the outer membrane of *Rb. sphaeroides* a Mr 21,500 protein with altered electrophoretic mobility after lysozyme treatment, suggesting an association with peptidoglycan. They also found an Mr 8,000-10,000 lipoprotein that may be equivalent to the BLP of *E. coli*.

### 1.2.6 Extracellular layers

External to the outer membrane of some species lies a regularly arranged layer of protein particles (Glauert and Thornby, 1969). Such a layer has been isolated from *Rhodospirillum rubrum* (Evers *et al.*, 1984). In this species a single Mr 68,000 protein was identified by both surface iodination and sucrose wash-treatment, and was found to be the

sola component of the surface array.

Many genera of bacteria include species capable of producing polysaccharides outside the cell wall. These exopolysaccharides (EPS) may take the form of a discrete capsule and/or an extracellular slime apparently unattached to the bacterial surface (Sutherland, 1977). The exact chemical nature of the link or links between polysaccharide and wall polymers and the method of transportation of the EPS to its extracellular location are still unknown. In certain species of the *Rhodospirillaceae* such as *Rb. capsulatus* (Flammann *et al.*, 1984a; Weaver *et al.*, 1975) polysaccharide capsule and slime layers are found external to the outer membrane. These have been visualised by electron microscopy using specific antisera (Omar *et al.*, 1983a; Flammann *et al.*, 1984b).

### 1.3 Bacteriophage

#### 1.3.1 Classification of bacteriophage

Bacterial viruses or bacteriophages, were first discovered by Twort in England and independently by d'Herelle in France, nearly half a century ago. Viruses in general vary widely in their size and shape, in their location within the cell (eukaryotic viruses), where they start to multiply, in the type of nucleic acid (DNA or RNA) which they contain, and are classified according to these criteria.

Four types of nucleic acid have been identified in bacteriophages, double stranded DNA (2-DNA), single stranded DNA (1-DNA), double stranded RNA (2-RNA), single stranded RNA (1-RNA), and although

bacterial viruses exhibit great diversity they fall conveniently into six basic morphological groups. Bradley (1967) proposed a scheme of classification based on previous observations (Fig. 3), and suggested that morphological groups A, B and C could, if required, be further divided on the basis of head shape and tail morphology.

As a consequence of the lack of morphological or chemical criteria for classification, categories of phage have also been distinguished, on the basis of their interaction with the host bacteria, as virulent or temperate (section 1.3.3 and section 1.3.5). Although the distinction between virulent and temperate phage is convenient, it is of little taxonomic use since a virulent phage can arise from a temperate phage as a result of single mutational event.

Currently, the International Committee on Taxonomy of Viruses (ICTV) use the following criteria for the classification of phage without hierarchical order: (i) nucleic acid: nature, mass and composition; (ii) virion: symmetry, dimensions, mass and gross composition; (iii) antigenic relationships: host range and resistance against the environment (chiefly ether, chloroform and heat) (Ackermann, 1987).

### 1.3.2 Morphology

Most, if not all, viruses are built to the same principles, i.e. they are in the form of a nucleic acid core surrounded by a protein coat or capsid. As shown in Figure 4a, the shape and size of the protein coat varies greatly ranging from filamentous to others with a variety of distinct head and tail structures, and some, though rare, surrounded by a lipid layer, i.e. enveloped.



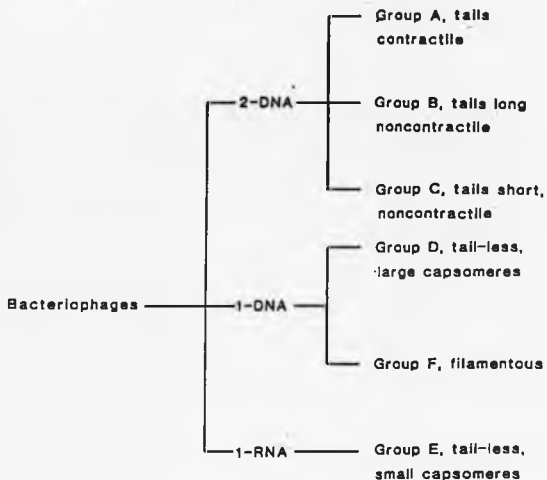


Figure 3

Bradley's scheme of classification of bacteriophage

Figure 4a

Schematic diagrams of the shapes and relative sizes of known bacteriophage (Matthews, 1979)

Figure 4b

Diagram showing the morphological components of phage T2, and their arrangement in the intact structure (Hayes, 1968)



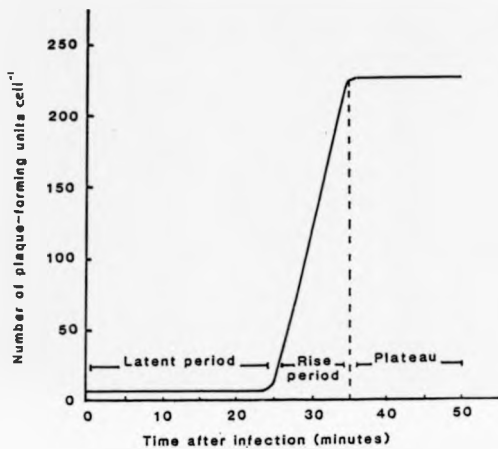
By far the most intensive studies on the structural components of bacteriophage, and the way in which these are assembled to form a co-ordinated infective particle have been made on the T-even phages and on T2 and T4 in particular (Mathews *et al.*, 1983). Figure 4b shows the structure of T2. The head has the form of an elongated bipyramidal, hexagonal prism about 1000 Å long and 650 Å wide and is attached to a straight tail about 1000 Å long and 25 Å wide. The volume of the phage is therefore approximately one thousandth that of its bacterial host. Phage particles become attached to bacteria by means of their tails (Anderson, 1953). This adsorption can be seen to be accompanied by a striking change in the appearance of the phage tail, the upper part of which becomes contracted and thickened to reveal a protruding core about 70 Å in diameter. That part of the tail which undergoes contraction is known as the sheath. At the distal end of the core is a hexagonal base plate from which project six spikes and six long tail fibres each 1300 Å in length and 20 Å wide (Hayes, 1968).

#### 1.3.3 The life cycle of a lytic phage

The character of virulent phages is invariably to lyse the bacteria they infect; they have no alternative but to follow what is termed the lytic cycle. The lytic cycle follows a sequence of events first described by D'Herelle: (1) infection of a few bacteria in a culture by a few virus particles in the inoculum; (2) intracellular multiplication of the infecting viruses to yield an issue of more progeny viruses; (3) release of these progeny by lysis of the infected bacteria; (4) reinfection of a greater number of bacteria by the progeny; (5) repetition of the intracellular-multiplication-lysis-reinfection cycle, until a number of phage particles have been produced sufficient to infect and finally lyse all of the cells of the culture.

Ellis and Delbrück (1939) devised a one-step growth experiment to study the kinetics of phage multiplication in bacterial populations. An inoculum of phage suspension is added to a mid-exponential culture of sensitive bacteria. After a few minutes, during which particles become adsorbed to and infect the bacteria, residual free phage must be removed to prevent further infection. If a phage shows a specific cation requirement for adsorption cells may be diluted into media minus the essential cation. Alternatively, free phage particles may be neutralised by the addition of antisera, the "critical site" for neutralisation residing in the phage tail (Adams, 1959).

A typical one-step growth curve is given by the example for phage T2 in Figure 5. As can be seen, the number of plaque forming units in the culture remains constant for the first 25 min after infection. This initial interval during which the infective titre shows no increase is the latent period. After a further 10-20 min the plaque count rises sharply to reach a plateau. The time interval during which the number of plaque forming units increases is the rise period, and the ratio of the final titre of the plateau to the initial titre of phage-infected bacteria is the burst size. The latent period thus represents the time which elapses between the moment at which the bacterial culture is infected with phage and the moment at which the first infected cells in the culture lyse to liberate progeny phage particles. Evidently, the plaques produced upon plating aliquots of the infected culture during the latent period are not derived from individual free phage, but instead from phage infected bacteria containing progeny phage. Only after the latent period, when the intracellular phages have escaped from the host cell into the culture medium can each progeny phage form its own plaque and hence the rise period is generated. The plateau is



**Figure 5**

A theoretical graph illustrating the one-step growth experiment

achieved when all the infected bacteria have burst and released their content of phage. These free particles are prevented from adsorbing to uninfected bacteria by the low population density of the latter in the diluted mixture.

If all of the infected bacteria burst at the same time, the increase in the plaque titre to plateau level would be instantaneous. The rise period therefore represents the scatter in burst times from individual phage-bacterium complexes. This is due partly to variation in the initial adsorption time which leads to asynchrony in the onset of phage growth. Since phage multiplication depends on bacterial metabolism, synchronisation can be achieved by temporarily poisoning cells with cyanide (Denhardt and Sinsheimer, 1965) or chloramphenicol (Sinsheimer et al., 1967). However, the concentration required for the latter is not always easy to determine.

Both the latent period and the burst size vary widely with the strain of phage and bacterium used and the physiological state of the bacterium.

The example of the phage T2/*E. coli* system indicates the experimental stages involved in a typical one-step growth curve determination but over-simplifies the problems incurred when other phage/bacterium systems are studied. The fact that protocols can differ so greatly, e.g. the use of cations, or antibodies and/or cell synchronisation, results in difficulty in the comparison of data between different phage-bacterium systems and laboratories.

#### 1.3.4 The infective process

The process of phage infection may be arbitrarily divided into a number of stages: adsorption, penetration and intracellular development, maturation and lysis.

##### 1.3.4.1 Adsorption and receptors

The first step in adsorption prior to infection, is a random collision between the phage and bacterium. The extent to which adsorption occurs in any mixture of phage and bacteria can be determined in several ways. The most common method is to centrifuge the mixture at low speed, so that only the bacteria, but not the free phage particles, are pelleted. The supernatant can then be titred for free phage.

The extent of adsorption depends not only on the strains of phage and bacteria but also upon the composition of the medium in which the two bodies encounter each other. In particular, the pH and ionic composition play an important part (Adams, 1959). Inorganic salts can act to neutralise the net negative charges on the bacterium and phage and thus facilitate initial contact (Puck and Tolmach, 1954). Cations may also bring about some subtle structural changes in the phage required for the adsorption process (Hayes, 1968). Anderson (1945) discovered that coliphage T4 and T6 are unable to adsorb to their host cells unless certain organic compounds are present in the environment. He determined by testing various substances, that L-tryptophan or L-phenylalanine resulted in a great increase in the efficiency of plating. In phage T4 the tail fibres can retract to form a jacket around the sheath in the absence of tryptophan and extend in its presence to allow interaction with the cell surface (Brenner *et al.*, 1962).



For attachment of a phage particle to the bacterial surface to lead to infection, adsorption must become irreversible. Firm chemical bonds must be formed between the cell surface and virus and moreover, as with T6 adsorption, this can be accompanied by a striking change in structure preventing desorption (Mathews *et al.*, 1983).

Adsorption of phage tends to be highly host-specific suggesting that bacteria must contain some phage-specific receptor sites that are capable of precise and durable interaction with the phage tail. Almost every structure on the surface of a bacterial cell, or extending from it can act (or include) phage receptors. Phage can adsorb to structures as varied as flagella, pili, capsules (EPS), lipopolysaccharide and surface proteins. A number of reviews extensively discuss the nature of bacterial receptors (Lindberg, 1973; Lindberg, 1977; Braun and Hantke, 1977). In brief:

a) Protein receptors

Receptor activity for phage T6 was found in phenol-soluble extracts of bacteria presumably containing the outer membrane. It was inferred that the receptor sites were proteins and this was confirmed by a number of independent observations. These included that the inactivating capacity of the outer membrane was abolished by treating with proteolytic enzymes, and that purified LPS was not capable of inactivating T6 (Micheal, 1968). Manning and Reeves (1976) found that a Mr 25,000 protein was missing from T6 resistant mutants and it was subsequently shown that this protein was an important component for nucleoside transport in whole cells and a receptor for colicin K (Hantke, 1978). Many other known phage receptor proteins of *E. coli* K12 have been shown to be multifunctional, i.e. involved in substrate uptake and receptors

for phage and colicins. For example, phages T5 and T1 adsorb to the Mr 78,000 protein (Ton A) involved in ferrichrome uptake and a receptor for colicin M (Braun and Hantke, 1977) and phage  $\lambda$  to Lam B a Mr 55,000 protein, essential for maltodextrin transport (Hazelbauer, 1975).

b) LPS receptors

Bacteriophage adsorption to, and characterisation of, LPS and EPS receptors of Gram negative bacteria has been reviewed in detail by Lindberg (1977). As discussed in section 1.2.4., LPS consists of three regions, the lipid A moiety, the R-core and the O-antigen. The host range of phage where the receptor is located in the O-antigenic side chain is relatively narrow. This is partly due to the large variation in the structure of the O-antigenic polysaccharide seen in Gram negative bacteria. Edward and Ewing (1972) found more than 350 O-antigens in *E. coli*, *Shigella*, *Salmonella*, *Klebsiella*, *Enterobacter* and *Serratia*. Several phage for *Salmonella* are temperate and can cause lysogenic conversion, i.e. the appearance of new O-antigenic specificities (Barksdale and Arden, 1974). Most, if not all, of these phages belong to morphological group C according to Bradley's scheme of classification. A number of phage are active on both smooth and rough strains (section 1.2.4) and others are active on rough strains of the host only. The F0 phage lyse most smooth *Salmonella* strains and all rough strains with a complete basal core (Lindberg and Hallerquist, 1971). The rate of attachment of phage F0 to smooth strains is slower than that to rough strains, probably due to O-side chains causing steric hindrance and therefore limiting access of the phage to its receptor in the core (Lindberg and Holme, 1969).

Phages T3, T4 and T7 are thought to be rough specific. The evidence for this is that, (i) *E. coli* B and *E. coli* K12 are rough mutants (whose smooth parent strains are unknown); (ii) *Shigella sonnei* phase I bacteria are resistant, whereas the phase II bacteria, which are susceptible, possess characteristics which classify them as rough mutants; (iii) smooth strains of *Sh. flexneria* are resistant to phage T3, T4 and T7 but their spontaneous rough mutants are sensitive to one or more phage (Lindberg, 1977).

c) EPS receptors

As discussed briefly in section 1.2.6 bacteria may be surrounded by a capsular or less well defined slime exopolysaccharide layer. This layer may serve for attachment of some phages, but it can also block the access of bacteriophage to the receptor present in the underlying cell wall structure. Most phages that display a lytic activity against capsular polysaccharides belong to Bradley's group C, i.e. have a very short non-contractile tail (Stirn and Feund-Mölbart, 1971). In addition, the capsule-specific phage do not seem to be irreversibly inactivated by isolated capsular material. This suggests that the capsular layer serves as a site for reversible attachment only and that other cell wall components are required for subsequent irreversible attachment (Stirn *et al.*, 1971).

d) Protein/LPS receptors

The above description of protein, LPS and EPS receptors as discrete receptors is perhaps an over-simplification, suggesting a lack of interaction between each component. However, in *E. coli*, Omp C and Omp F, porin proteins are receptors for a number of phage including T4, Tu1b, Me1 and Tu1a. However, when the isolated porins were suspended in

Triton X-100 in bicarbonate buffer, or complexed with phospholipids, the porins did not inactivate these phages, and reconstitution of the receptor activity required the addition of LPS (Van Alphen *et al.*, 1979; Datta *et al.*, 1977). Yu *et al.* (1981) showed that lipid A did not substitute for LPS in the reconstitution of Tu1b receptor, and that LPS isolated from a heptose-less LPS mutant of *E. coli* was very poorly active in reconstitution. These results indicated that Omp C porin must interact with the core region of LPS to undergo a conformational change, before it can act as a phage receptor. Alternatively, Omp C protein and LPS may constitute two independent parts of the receptor complex, both of which are essential for the inactivation of Tu1b.

A different system exists in the case of the receptor for phage T4.

Phage T4 is inactivated by the LPS isolated from *E. coli* B, however, LPS isolated from *E. coli* K12 requires the presence of the Omp C protein (Yu and Mizushima, 1982). In *E. coli* B the core oligosaccharide terminates in a glucose region which is thought to be the T4 receptor site.

However, in *E. coli* K12 this glucose region is masked by additional glucose and galactose residues which may prevent the LPS interacting with the tail fibre of T4. Yu and Mizushima (1982) determined that the removal of the additional residues results in the appearance of B-type receptor activity in the K12 strain. Further results also suggest that the Omp C protein and the glucose region of LPS in T4 infection can be replaced by one another.

#### Models for phage adsorption to bacterial surface carbohydrates

Adsorption of a phage to its receptor on the cell is, in most instances, followed by penetration of nucleic acid through the cytoplasmic membrane. Lindberg (1977) describes in detail a number of systems phage

have developed to overcome the problem of how adsorption, which sometimes takes place far away from the cytoplasmic membrane, is almost invariably followed by nucleic acid penetration. Lindberg (1977) proposed models for all types of receptors discussed above. With the possible exception of  $\phi$ X174, adsorption takes place by the formation of multivalent bonds between cell surface components and phage appendages such as the long tail fibres and tail spikes. Injection of DNA from the phage particle only occurs after cell-phage contact and bonding. In the case of phage with binding sites in the capsule, or O-antigen chain, this occurs only after the phage, through enzymatic hydrolysis of the binding site, has reached the cell wall.

#### 1.3.4.2 Penetration

Hershey and Chase (1952) proved with isotopically labelled phage T2 and *E. coli* that only the phage DNA penetrates the infected bacterium, and that the protein coat remains outside. However, this is not exclusively so, as approximately 3% of the total phage protein is injected with the DNA. This protein is not part of the phage envelope but is comprised of polyamines, spermidine and acid soluble polypeptides (Ames *et al.*, 1958) which function to neutralise the acidity of the DNA and form a matrix which promotes its condensation. An important observation as to how penetration occurs was the discovery that tails of the T-even phage (Weidel and Primosigh, 1958) and phage  $\lambda$  (Hendrix *et al.*, 1983) contain a lysozyme-like enzyme which acts on the peptidoglycan in the cell wall. There is evidence that some of the substances released by the enzyme action can trigger the discharge of phage DNA, even in the absence of cell walls (Garen and Kozloff, 1959).

The current model is as follows: The phage tail interacts with the cell wall and is brought into contact with zinc complexes, resulting in the extension of the tail fibres. Simultaneously, the tail makes contact with the wall allowing the enzyme to rupture at a localised point of the rigid layer of the wall. The products of this lytic activity trigger the contraction of the sheath and penetration of the cell wall by the core allowing the DNA to be discharged.

#### 1.3.4.3 Intracellular development

Doermann (1952) devised an experiment to follow the intracellular events after phage infection. During the course of a one-step growth experiment, with phage T4, he broke open the infected cells at intervals during the latent period. The latent period could be divided into two periods. The first was the eclipse, when no infective phage were found. The phage existed in a state different from that of the infecting particle; this non-infective form of a phage is known as a vegetative phage. During the second phase, called the maturation period, complete infective phage accumulate within the infected cells before being released by cell lysis.

Various classes of novel enzyme activities may appear after phage infections but only the largest phage such as  $\lambda$  and the T-even phage, produce all classes (Luria, 1978). One class consists of enzymes that are capable of abolishing host functions, for example nucleases. These either attack the host cellular DNA or catalyse certain initial steps in the replication and maturation of the phage genome. A second class consists of enzymes that catalyse the biosynthesis of precursors for phage nucleic acids, for example T-even phage contain 5-hydroxymethylcytosine in place of the cytosine found in *E. coli* cellular DNA (Mathews

et al., 1983). A third class of phage initiated enzymes is specifically related to the replication and expression of the phage genome, for example DNA polymerase, RNA replicases and RNA polymerase. In some cases phage encodes a completely new RNA polymerase, e.g. T3 and T7 code for an RNA polymerase consisting of a single polypeptide chain of approximately Mr 110,000 (Losick and Chamberlin, 1976). Often, however, phage produce protein subunits (sigma factors) that confer new specificities to the host RNA polymerase. Doi and Lin-Pa (1986) have described in detail the replacement of sigma ( $\sigma$ ) factors from *Bacillus subtilis* RNA polymerase by  $\sigma$  factors synthesised by the phage SP01.

#### 1.3.4.4 Maturation and lysis

When intracellular precursor pools of DNA and protein attain a certain size, infective phage particles begin to appear and increase in number at the same linear rate as the total DNA and protein pools. Thus, the pool sizes remain constant. From phenotypic mixing experiments (Stent, 1963), it was apparent that the protein parts of phage particles are assembled randomly. Kellenberger (1961) reviewed in detail electron microscopic observations on the maturation of phage particles. One of the first steps in phage maturation is the condensation of the phage DNA which is dependent on phage encoded proteins. Although evidence suggests that some structural protein components of the phage coat are capable of self-assembly in phage T4 and  $\lambda$  the overall situation is more complicated. For example, the head protein subunit of phage T4 is determined by a single gene (no. 23) but the shape of the capsid formed by "self assembly" of these subunits can be profoundly modified by mutations in other genes (Mathews et al., 1983). It is still not clear how DNA is taken up and condensed inside the capsid.

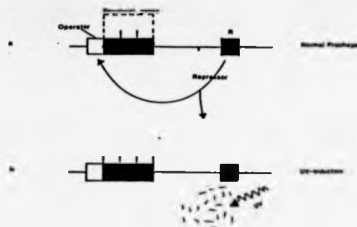
As described above in section 1.3.3 the latent period of the one-step growth curve ends with the bursting of the infected bacteria and the liberation of progeny phage. Liberation of phage  $\lambda$  requires two proteins, an enzyme that appears to damage or digest the cytoplasmic membrane and an endolysin that digests the rigid peptidoglycan layer of the cell envelope (Hendrix *et al.*, 1983). A similar enzyme is found with phage T4 (Mathews *et al.*, 1983). These endolysins differ from a true lysozyme in that they hydrolyse a peptide bond, whereas lysozyme splits peptidoglycan at adjacent N-acetyl muramic acid (NAM) residues. One noticeable exception to the lysis of cells and subsequent release of progeny phage are the filamentous coliphage, e.g. phage fd. Infected bacteria do not lyse, instead they continue to grow and divide with the filamentous progeny particles being extruded longitudinally through the cell wall (Hayes, 1968).

#### 1.3.5 Temperate bacteriophage and lysogeny

Evolution has given phage a variety of mechanisms for survival. So far, only the lytic life cycle of phage has been described, but another major form of phage cell interaction does exist. A temperate bacteriophage can be transmitted either from cell to cell via the infective lytic cycle or passed from mother to daughter within a cell line. The latent phage genome in such a cell line is called a prophage and the cells harbouring the prophage are termed lysogenic. Two outstanding properties of lysogenic bacteria are that firstly they carry as a stable heritable trait the potential to produce and release phage, and secondly, that they are immune to lytic infection by the same or closely related phage (Hayes, 1968). Barkadale and Arden (1974) reviewed lysogeny and its effect on the host bacterium. One such effect has been discussed previously - lipopolysaccharide conversion (section 1.3.4).



Stable lysogeny requires silencing of the expression of many genes related to the lytic cycle of the phage by a repressor protein binding to the operator region of these genes. Under certain conditions such as exposure to ultra-violet light, mitomycin C and shifts in temperature the repressor protein is broken down allowing expression of the lytic genes and induction of the phage (Hayes, 1968).



The prophage may be integrated into the bacterial chromosome, as with  $\lambda$  (Hendrix *et al.*, 1983) or exist like an independent plasmid, e.g. phage P1 (Luria, 1978). Temperate phages fall into three classes with regard to UV induction (Barksdale and Arden, 1974): a) those that are readily inducible by UV such as coliphage  $\lambda$  or corynebacteriophage  $\beta$ ; b) those which show a slight inducibility following irradiation, e.g. coliphage P1; c) those which are not induced at all, as in the case of coliphage P2 (Bertani and Bertani, 1971) and bacteriophage Mu (Howe and Bade, 1975).

### 1.3.6 Phage of the Rhodospirillaceae

Important features of phage that have been isolated for members of the *Rhodospirillaceae* and characterised are listed in Table 1. Little is known about the bacteriophage of photosynthetic bacteria. Virulent phages have been isolated for several purple non-sulphur bacteria, namely *Rp. palustris* (Rp1), *Rb. capsulatus* (RC1) and *Rb. sphaeroides* (RS1) (Table 1). A number of temperate phages have been isolated for *Rb. sphaeroides* (RSG, RSGP, RSG1 (Table 1)). It has been shown that of seventeen natural isolates of *Rb. sphaeroides* isolated from stagnant ponds in Australia, all possessed at least one and up to three prophage (Femberton *et al.*, 1983). In contrast, all 95 phage isolated by Wall *et al.* (1975) were only virulent against strains of *Rb. capsulatus*.

As yet no phage capable of transduction has been isolated for the *Rhodospirillaceae*. Nevertheless, a form of generalised transduction does occur in *Rb. capsulatus* (Solioz *et al.*, 1975) which is mediated by a non-infective, small phage-like particle described as a gene transfer agent (GTA).

As can be seen from Table 1, the phage of the *Rhodospirillaceae* have varying morphologies belonging to Groups A, B and C of Bradley's classification (section 1.3.1). Phage RS1 and phage RC1 have specific cation requirements for adsorption of magnesium and calcium and magnesium respectively. Rp1, a phage for *Rp. palustris* (Freund-Mölbart, 1968) adsorbs to specific sites on the host cell surface (Bosecker *et al.*, 1972b). The adsorption of phage Rp-1 was shown to be clearly dependent on the age of the cell and specific for the actively growing pole and plane of division of the cells. It was concluded that changes in the cell surface occur with ageing of the cells and these prevent

Table 1

Host	Phage	Type	Morphology	Comments	References
<u>Rp. palustris</u>	Rp1	virulent ds-DNA	Icosahedral head, short tail	shows specific adsorption to new pole and plane of division between cells	Freund-Molbert <u>et al.</u> , 1968 Bosecker <u>et al.</u> , 1972a Bosecker <u>et al.</u> , 1972b
<u>Rb. sphaeroides</u> 2.4.1	RS1	virulent ds-DNA	65nm poly- hedral head, long tail 65nm	DNA shows size heterogeneity, G+C content of DNA 46 mol % Mg <sup>2+</sup> , Ca <sup>2+</sup> cations required for adsorption	Abeliovich & Kaplan, 1974 Donohue <u>et al.</u> , 1985
	R01	temperate ds-DNA	polyhedral head, long tail	induced by mitomycin C	Mural & Friedman, 1974
	ØRsc1	temperate ds-DNA	hexagonal head 90x40.5nm, long tail 116nm. collar present	mitomycin C induced G+C content of DNA 71.8 mol %	Duchrow <u>et al.</u> , 1985 Duchrow & Giffhorn, 1987

(...Contd.)

Table 1 ....(contd.)

Host	Phage	Type	Morphology	Comments	References
<u>Rb. sphaeroides</u> 601	R06P	temperate ds-DNA	polyhedral head, long tail	exists as a plasmid during lysogeny, confers penicillin resistance G+C content of DNA 63.5 mol %. Induced by mitomycin C.	Pemberton & Tucker, 1977 Tucker & Pemberton, 1978
	R09	temperate ds-DNA	indisting- uishable from R06P	acts as a helper phage in transfection with R06P DNA. Mitomycin C induced.	Tucker & Pemberton, 1980
<u>Rb. capsulatus</u>	RC1	virulent ds-DNA	polyhedral head, tail sheath, contractile, base plate with tail fibres	requires Ca <sup>2+</sup> for adsorption, used in studies of the bio- energetics of phage replication	Schmidt <u>et al.</u> , 1974
<u>Rb. capsulatus</u>	Gene transfer agent (GTA)	ds-DNA	small, 30nm icosahedral head, very short tail	not classified as true phage wide spread occurrence, contain 2-3 Kb of DNA	Wall <u>et al.</u> , 1975 Solioz <u>et al.</u> , 1975

adsorption of the phage particles over the entire cell surface. Phage Rpl was found to be capable of adsorbing to intra-cytoplasmic membrane isolated and purified from its host. This was later attributed to contamination of the intra-cytoplasmic membrane by lipopolysaccharide complexes which function as the receptors for this phage (Rosecker et al., 1972b).

Schmidt et al. (1974) studied the energy requirement for replication of the virulent bacteriophage RCl in *Rb. capsulatus*. As discussed in section 1.1 the host bacterium can obtain energy either by photophosphorylation (anaerobic growth) or by oxidative phosphorylation (aerobic growth in the dark). Thus, changes in the mode of growth affect the relative capacities of the energy converting systems. These capacities are in turn reflected in the ability of infected cells to support virus growth. Thus, the non-sulphur bacteria/phage systems offer a unique system to study the bioenergetics of phage growth.

Flammann and Weckesser (1984c) studied the cell wall composition of phage resistant mutants of *Rb. capsulatus* St. Louis RCl<sup>-</sup> in an attempt to determine the receptor for phage RCl adsorption. There appeared to be no differences in the lipopolysaccharide, peptidoglycan and composition of the cell wall fractions between mutant and wild type cells. The only difference apparent was the absence of a firmly bound capsule in *Rb. capsulatus* St. Louis RCl<sup>-</sup>.

Abeliovich and Kaplan (1974) characterised in detail the bacteriophage RS1 specific for *Rb. sphaeroides* 2.4.1. Donohue et al. (1985) later noted a considerable size heterogeneity and free 3' hydroxyl groups present in several distinct regions of RS1 DNA.

R86P, a temperate phage specific to *Rb. sphaeroides* 6.0.1 (Tucker and Pemberton, 1978), is unique among the *Rhodospirillaceae* phage in that after lysogenisation (section 1.3.5) it exists as an extrachromosomal plasmid. Tucker and Pemberton (1980) found that *Rb. sphaeroides* could be transfected by bacteriophage R86P DNA even when the recipient cells were lysogenic for R89.

### 1.3.7 Uses of phage

Bacteriophage have proved to be invaluable in studies involving many aspects of microbiology, but of singular importance has been their contribution to the development of molecular biology, particularly in its infancy. Phage systems proved to be ideal models to help elucidate some of the basic problems faced by molecular biologists, such as the genetic code. The realisation that the assembly of protein subunits into a functional unit does not necessarily require further enzyme activity came from studies on the self assembly of phage proteins *in vitro*. Phage nucleic acids have also proved to be useful templates for studying the kinetics of RNA polymerase binding and transcription. Studies on the temporal regulation of phage genes have given an insight into control mechanisms involved in gene expression and regulation. Phage have also proved useful in the identification and typing of bacteria and in systematics, their host range can give an insight into taxonomic relatedness. The main significance of phage today is undoubtedly the importance it has maintained as a genetic tool in molecular biology. Phage are well documented as adaptable vectors for gene cloning and manipulation, and are useful in strain construction via techniques such as transduction and transfection. Three of the uses of phage relevant to this project are described below.

#### 1.3.7.1 Transduction

Perhaps the most widely used means of effecting genetic exchange in bacteria has been transduction, i.e. gene transfer mediated by bacteriophages. In specialised transduction, which is observed only with temperate phage, occasional prophages are excised and packaged together with adjacent segments of bacterial DNA which on subsequent reinfection can be incorporated into the recipient's genome through gene recombination. A well documented example is that of phage  $\lambda$  (Stahl and Weisberg, 1983). Upon lysogenisation of *E. coli* K-12, the prophage always inserts at the same site on the bacterial chromosome between the *gal* and *bio* genes and in the same orientation. In a lysate prepared by inducing this lysogen, about one  $\lambda$  particle in  $10^5$  includes DNA from the nearby *gal* operon.

In generalised transduction, which can be mediated by both virulent and temperate phage, any region of the bacterial chromosome can be transferred by the phage particle. During phage development in the host cell, the host's chromosome is fragmented, then during maturation, a small proportion of the phage capsids incorporate fragments of the bacterial chromosome in place of phage DNA, e.g. phage P1 and P22 (Luria, 1978).

The usefulness of transduction in the mapping of bacterial genes is well established. In specialised transduction, because the bacterial DNA packaged is only a small proportion of the bacterial genome, it is possible to map regions of bacterial DNA by deducing linkages from the transduction data. The further two linked markers are from one another, the less frequently they are co-transduced. Generalised transduction has also been useful for strain construction.

#### 1.3.7.2 Transfection

Kaiser and Hogness (1960) first demonstrated the infection of bacteria by isolated bacteriophage nucleic acid and the subsequent production of normal phage progeny. This is termed transfection. When a preparation of wild type phage  $\lambda$  DNA was added to sensitive *E. coli* bacteria which had been previously infected with defective, mutant  $\lambda$  particles which by themselves are unable to produce plaques, the wild type DNA molecules were taken up by the cells, and gave rise to active infection and plaque formation. The phage DNA alone was not infective and required the addition of the mutant 'helper' phage to penetrate the cell. Temperate bacteriophage have also been implicated in the development of cells that can take up DNA, i.e. competence, in other bacterial species. For example, P11 was a requirement for competence in *Staphylococcus aureus* and addition of viral DNA to the lysogenic cells increased the level of transfection. Also, strains of *Bacillus subtilis* 168, lysogenic for bacteriophage  $\phi$ 105, had an increased level of transfection with viral nucleic acid as compared with non-lysogenic strains. However, the frequency of chromosomal markers, i.e. transformation, was decreased by lysogeny and superinfection by phage could not increase the level of transformation (Tucker and Pemberton, 1980). Subsequently, *B. subtilis* has been found to be readily transfected by DNAs from many of its phages. Moreover, fragmented phage genomes can give rise to productive infection by recombination when several molecules are taken up by the same cell.

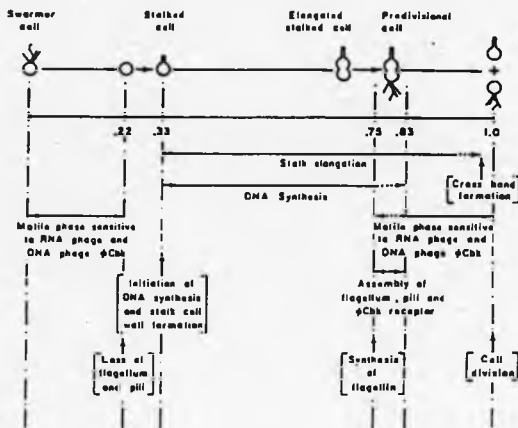
#### 1.3.7.3 Phage as cell surface markers

The use of phage as markers for macromolecular changes on the cell surface of bacteria has been applied in particular to *Caulobacter crescentus* and its phages. Relatively few bacteria carry out a defined



morphogenesis other than cell division, during their life cycles. Examples include, apart from *Caulobacter*, *Hyphomicrobium* and some members of the *Rhodospirillaceae*, in particular, *Rhodomicrobium vannielii* and *Rhodopseudomonas palustris*. The *Caulobacter* cell cycle includes at least two cell types and is characterised by the expression of temporally distinct morphological events (Shapiro, 1976) (Fig. 6). Asymmetric cell division and the formation of structures at the cell pole results in a unique polarity which is maintained throughout the cell cycle. The motile swarmer cell is a transitory cell with a polar flagellum and pili. The swarmer becomes a stalked cell by shedding its flagellum and synthesising a stalk at the same site. The stalk is formed by the extension of the cell wall and membrane at the cell-stalk junction and therefore represents localised cell wall synthesis. While both cell and stalk are increasing in length the cell expresses a phenotypic polarity. Binary fission of the elongated cell yields dissimilar daughter cells - both morphologically and physiologically. Prior to division a new flagellum and pili are assembled at the swarmer pole. Recently, Nathan *et al.* (1986) observed differential localisation of membrane receptor chemotaxis proteins in the *Caulobacter* predivisional cell. Similarly, Koyasu *et al.* (1983) found that different penicillin-binding proteins were located in isolated stalks compared to those of total cell envelopes, raising the question of how these proteins are targeted to distinct membrane sites within the *Caulobacter* cell.

Studies on *Caulobacter* mutants that are blocked at various stages of morphogenesis have helped towards an understanding of the relationship between gene function and the series of events in differentiation. In order to easily identify differentiation mutants, Shapiro *et al.* (1971)



**Figure 6** Schematic representation of *Caulobacter crescentus* cell cycle events taken from Shapiro (1976)

developed a differentiation assay based on several *Caulobacter* phage.

ØCb5 (Bandis and Shapiro, 1970) is a small RNA bacteriophage which specifically adsorbs to pili present on the *Caulobacter* swarmer cell, and susceptibility of the host cell to phage infection is therefore limited to a specific cell and at a specific point in the differentiation cycle. The DNA bacteriophage ØCbK was shown to specifically infect the swarmer cell or a predivisive form with swarmer cell characteristics (Agabian-Keshishian and Shapiro, 1970). Initial evidence that phage adsorption required a specific host property came from one-step growth experiments with synchronised populations. A longer eclipse period was observed with the stalked cell than with the swarmer cell, suggesting that time was required for the formation of the sensitive cell type. The receptor site apparently resides in the cell wall lipopolysaccharide.

The RNA and DNA bacteriophage have proved useful in studying differentiation in *Caulobacter* as the phage receptor sites are synthesised at specific times in the cell's life cycle. Using labelled ØCb5 and synchronised swarmer cells, the time required for the transition from swarmer to stalked cells was deduced to be 30 min. This is the time at which the loss of cell motility is observed microscopically. Similarly, labelled ØCbK was added to synchronised stalked cells and an increase in phage adsorption was noted between 50 and 60 min. Thus, structural changes in the cell wall of stalked cells preparing for cell division could be observed and defined by a simple radiochemical assay.

Begg and Donachie (1977) used bacteriophage T6 as a marker to follow the growth of the outer membrane in a strain of *E. coli* temperature sensitive for the production of the T6 receptor protein. This is only one of the many ways in which the cell surface of *E. coli* has been labelled to follow cell division. The results after radiolabelling, i.e. lipids or total membrane proteins, were different from those obtained where peptidoglycan was specifically labelled with radiolabelled diaminopimelic acid. Problems were encountered with the interpretation of data to determine the mode of cell wall growth during division, as only a few of the membrane proteins are fixed whereas other components are free to move in the membrane.

The T6 receptor protein (section 1.3.4.1) was thought to be permanently attached to peptidoglycan (Begg and Donachie, 1977), thus preventing lateral diffusion and making it a useful marker for studying cell division. However, Menchi and Buu (1986) have shown that only 50% of the T6-receptor protein (Fhu A) present in *E. coli* cells was associated with peptidoglycan.

#### 1.3.8 Aims

As discussed previously in Section 1.3.6, few bacteriophage have been isolated for members of the *Rhodospirillaceae*, and even fewer have been characterised to any extent. ØBHG1 is the first bacteriophage known to infect *Rhodospseudomonas blastica*, one of the more recently discovered members of the *Rhodospirillaceae*. The primary aim of this project is to characterise this novel bacteriophage and then study its interactions with its host under varying conditions. The information made available from these studies can then be used to compare ØBHG1 with the limited data available for phage of other photosynthetic bacteria.

Following an initial physical characterisation of ØBHG1 an analysis of the phage interactions with *Rp. blastica* cell surface receptors, and the mechanisms of phage replication under varying cell culture conditions will be made. Such studies may give an understanding of how ØBHG1 co-exists with its host under fluctuating environmental conditions.

As discussed in Section 1.1, *Rp. blastica* has an asymmetric life cycle and phage can be used to detect non-uniformity of the cell surface (Section 1.3.7.3). With information about the bacterial receptor - bacteriophage interactions it may be possible to use ØBHG1 as a marker for macromolecular changes on the cell surface of the bacterium, and ultimately use this to analyse the targetting of cell wall components to specific zones of the bacterium during growth.

It has also been discussed, in Section 1.3.6, that no bacteriophage for members of the *Rhodospirillaceae* has been shown capable of transducing host DNA fragments. Consequently, ØBHG1 will be tested for any transducing ability. Such a capacity would greatly aid the development of a genetics system for its host.

**MATERIALS and METHODS**

## 2.1 Strains and Culture Conditions

### 2.1.1 Strains

The strains used in this study are listed in Table 2.

### 2.1.2 Media

Members of the *Rhodospirillaceae* were grown routinely in pyruvate-malate salts medium (Whittenbury and Dow, 1977) supplemented with 0.1% (w/v) yeast extract (PMY).

	g l <sup>-1</sup> distilled water
Sodium hydrogen malate	1.5
Sodium pyruvate	1.5
NH <sub>4</sub> Cl	0.5
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.4
NaCl	0.4
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.05
Yeast Extract	1

For the growth of *Rm. vannielii* yeast extract was omitted. The pH of the medium was adjusted to 6.8-6.9 with potassium hydroxide (KOH) pellets prior to autoclaving at 121°C for 15 min. After cooling sterile 0.1M phosphate buffer (pH 6.8) was added aseptically to a final concentration of 5 mM. For solid media, Difco Bacto-agar was added (1.5% w/v).

*Rp. blautica* was also grown with the defined pyruvate malate salt medium (PM). The same salts were used as in the preparation of PMY, however, yeast extract was omitted. Following autoclaving and cooling, phosphate

Table 2.            Sources of Strains

<u>Species</u>	<u>Strain Number</u>	<u>Reference</u>
<i>Rhodopseudomonas blastica</i>	NCIB 11567	Eckersley & Dow (1980)
<i>Rhodobacter sphaeroides</i>	NCIB 8253	
<i>Rhodobacter sphaeroides</i> sub sp. <i>cordata</i>		Gest et al. (1983)
<i>Rhodopseudomonas palustris</i>	NCIB 8288	
<i>Rhodospirillum tenue</i>	ATCC 25093	
<i>Rhodospirillum rubrum</i>	NCIB 8255	
<i>Rhodobacter capsulatus</i>	NCIB 8254	
<i>Rhodopseudomonas gelatinosa</i>	NCIB 8290	
<i>Rhodocrobium vannielii</i>	RM5 (Warwick)	Whittenbury & Dow (1977)
<i>Rhodopseudomonas blastica</i> <sup>r9</sup>	resistant mutant to ØRHG1	(section 2.4)
<i>Escherichia coli</i>	K12	



buffer was added as for PMY, and 0.75 ml and 100  $\mu$ l of a filter sterilised vitamin and trace element solution, respectively, were added per 100 ml of PM medium.

<u>Vitamin Solution</u>		g l <sup>-1</sup>	
Nicotinic acid	1.54		(Stored at 4°C
para-aminobenzoic acid	0.462		in the dark)
Thiamine-HCl	0.154		
Calcium-Pantothenate	0.216		
Vitamin B <sub>12</sub>	0.00431		
D-biotin	0.00325		
<u>Trace Element Solution</u>		g l <sup>-1</sup> (10x)	
EDTA	5		
FeSO <sub>4</sub> ·7H <sub>2</sub> O	2		(Stored at 4°C)
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.1		
MnCl <sub>2</sub> ·4H <sub>2</sub> O	0.03		
H <sub>3</sub> BO <sub>4</sub>	0.3		
CoCl <sub>2</sub> ·2H <sub>2</sub> O	0.2		
CuCl <sub>2</sub> ·2H <sub>2</sub> O	0.01		
NiCl <sub>2</sub> ·6H <sub>2</sub> O	0.02		
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.03		

During this study *Ep. blastica* was also grown on R8AH (Drews, 1965) which contained the following:

	<u>g l<sup>-1</sup> distilled water</u>
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.2
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.07
EDTA	0.02
Sodium hydrogen malate	2.4
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	1.2
Yeast extract	0.96

In addition, 9.6 ml of Iron III citrate solution and 0.96 ml of trace elements were added.

Iron III citrate solution = 1.056 g per litre distilled water

<u>Trace elements</u>	<u>g l<sup>-1</sup></u>
MnSO <sub>4</sub>	0.02
H <sub>3</sub> BO <sub>3</sub>	0.01
(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub>	0.02
ZnSO <sub>4</sub>	0.01

The pH of the medium was adjusted to 6.8-6.9 with 20% (w/v) sodium hydroxide (NaOH) prior to autoclaving at 121°C for 15 min.

After autoclaving and cooling, 7.2 ml of filter sterilised vitamin solution and 10 ml of sterile 0.15 M potassium phosphate buffer pH 6.8 were added aseptically per litre of medium.

<u>Vitamin solution</u>	<u>mg l<sup>-1</sup> distilled water</u>
Biotin	10
Nicotine	266
Nicotinamide	266
Thiamine dichloride	523

*E. coli* was grown in Luria-Bertani (LB) medium containing 10 g Bacto tryptone, 10 g NaCl and 5 g yeast extract per litre of distilled water (pH 7).

### 2.1.3 Growth conditions

#### a) Small batch cultures

Liquid cultures of members of the *Rhodospirillaceae* were grown in 250 ml, conical flasks. For photosynthetic growth these flasks were sealed with rubber suba seals (William Freeman & Co. Ltd.) and flushed with oxygen free nitrogen for 15 min via sterile syringe needles inserted through the rubber suba seal. Flasks were then incubated in a 30°C shaking water bath with an incident light intensity of approximately 40  $\text{E}_m^{-1} \text{s}^{-1}$  provided by a tungsten lamp.

Chemoheterotrophic growth was achieved with flasks wrapped in silver foil and stoppered with cotton wool. These were shaken at 200 rpm at 30°C.

*E. coli* was grown aerobically in flasks at 37°C on a rotary shaker.

#### b) Fermenter batch culture

For large scale chemoheterotrophically grown batch cultures of *Rp. blastica* a fermenter (Microferm; New Brunswick Scientific Co) with a

12 L working capacity was used. Growth was on RS&H medium at 30°C with mechanical stirring (200 rpm) under strong aeration. 1% (v/v) antifoam (silicon Antifoam Emulsion 30, SERVA) was added automatically when required and the vessel was wrapped in a double layer of black material to exclude light. A 20 ml inoculum of an overnight culture of *Rp. blastica* was routinely used. Growth continued until an optical density of approximately 1.2 ( $A_{650}$ ) was reached, at which point the cells were harvested by continuous centrifugation.

#### 2.1.4 Maintenance of cultures

Culture purity was checked by phase contrast microscopy and streaking to individual colonies on PMY plates. Cultures were maintained by pelleting 10 ml of late exponential culture, washing once with 10 mM Tris-HCl (pH 7.3) and adding sterile glycerol to a final concentration of 15% (v/v). Cultures were vortexed and then stored at -20°C. It was found necessary to return to stock cultures of *Rp. blastica* when resistance to ØBHG1 increased with subculturing. Sensitivity to ØBHG1 was tested by cross streaking with  $10^8$  pfu ml<sup>-1</sup> and observing zones of lysis.

## 2.2 Phage Isolation, Purification and Titration

### 2.2.1 Phage isolation

ØBHG1 had been isolated from a small eutrophic pond using the method of Logan *et al.* (1981) and was donated by Dr. C. S. Dow.

### 2.2.2 Phage purification

A mid-exponential culture of *Rp. blastica* was infected with ØBHG1 to a multiplicity of infection (moi) of approximately 0.01. Flasks were left

shaking for 24 hr to ensure maximum lysis. A few drops of chloroform were then added and the flask left to shake for 20 min. Cell debris was removed by centrifugation in an MSE Chlapiin at 3,000 g for 10 min at 4°C. Caesium chloride was added to an average density of 1.546 g cm<sup>-3</sup>. Samples were ultra-centrifuged at 36,000 rpm (240,500 g) using a Beckman SW40 rotor and Beckman L8 ultra-centrifuge at 10°C for 36 hr, or at 26,000 rpm (121,900 g) for 36 hr using a SW28 Beckman rotor for larger preparations. The phage band, light blue in colour, was removed through the side of the centrifuge tube with a syringe and needle. These fractions were then pooled and dialysed against 2 l of phosphate buffered saline pH 7.0 (PBS) (NaCl, 8.0 g; KCl, 0.2 g; Na<sub>2</sub>HPO<sub>4</sub>, 1.15 g; KH<sub>2</sub>PO<sub>4</sub>, 0.2 g; l<sup>-1</sup>) at 4°C.

Further purification was performed by repeated addition of caesium chloride and ultracentrifugation. Phage were stored at 4°C in sterile containers.

#### 2.2.3 Phage titration

Serial dilutions of phage suspension were added to PHM top agar (PHM with 0.7% (w/v) Difco agar) containing 0.2 ml of an early stationary phase chemoheterotrophically grown *Rp. blastica* culture. Plates were routinely incubated at 30°C.

#### 2.3 Determination of Cell Numbers

To readily obtain an estimated viable count of *Rp. blastica* cells present per ml of culture, an optical density versus viable cell number graph was plotted (Figure 7). The optical density ( $A_{650}$ ) of serially diluted cultures of both photosynthetically and chemoheterotrophically

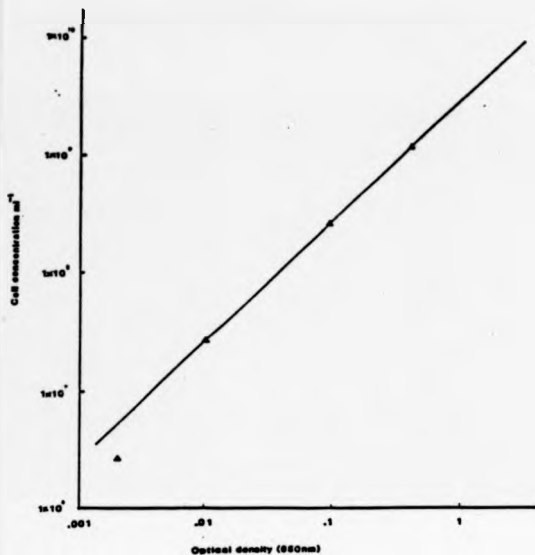


Figure 7

The relationship between optical density ( $A_{650nm}$ ) and the viable count of a chemoheterotrophically grown mid-exponential culture of *Rp. blasticus*

A photoheterotrophically grown culture of *Rp. blasticus* gave an identical result.

grown *Rp. blastica* (PMY) were plotted against the number of viable cells determined by spreading known aliquots onto the surface of PMY plates.

#### 2.4 Isolation and Enumeration of Spontaneous Phage Resistant Phenotypes of *Rp. blastica*

100  $\mu$ l of  $1 \times 10^{10}$  pfu  $\text{ml}^{-1}$  of  $\phi$ BHGl and 100  $\mu$ l of  $1 \times 10^9$  cells  $\text{ml}^{-1}$  of mid-exponential chemoheterotrophically grown *Rp. blastica* were spread together onto PMY plates. After 48 hr incubation colonies were counted and tested for phage resistance by cross-streaking with  $\phi$ BHGl. An alternative method for enumeration was by picking five individual sensitive colonies of *Rp. blastica*, which had been grown chemoheterotrophically in PMY to a concentration of  $5 \times 10^8$  cells  $\text{ml}^{-1}$ .  $\phi$ BHGl was then added to a moi of 10 and flasks left to shake for 20 min before 100  $\mu$ l aliquots were spread onto the surface of PMY plates. As above, colonies were counted and phage resistance tested after 48 hr incubation.

#### 2.5 Host Range of $\phi$ BHGl

Serial dilutions of  $10^{10}$  pfu  $\text{ml}^{-1}$  of  $\phi$ BHGl were spotted onto top agar lawns (PMY) of various members of the *Rhodospirillaceae* as listed in Table 2. Plates were incubated under photosynthetic and chemoheterotrophic growth conditions at  $30^\circ\text{C}$ .

#### 2.6 Adsorption Kinetics of $\phi$ BHGl to *Rp. blastica*

##### 2.6.1 Measurement of the rate of adsorption

$\phi$ BHGl was added to photosynthetically or chemoheterotrophically grown *Rp. blastica* to a known moi. The phage/cell suspension was incubated at

30°C without shaking. After various times a sample was carefully removed, serially diluted with cold PBS, then titred immediately for free phage.

#### 2.6.2 Measurement of the rate of irreversible adsorption

As above, ØBHG1 was added to *Rp. blastica* to a known moi, and the suspension incubated at 30°C. At various times a sample was removed and diluted 100-fold into cold PBS followed by centrifugation at 4,000 g in an MSE Chilapin at 4°C for 10 min. The supernatant was titred for ØBHG1.

#### 2.6.3 Determination of the optimum cell concentration for ØBHG1 adsorption

$5 \times 10^6$  pfu ml<sup>-1</sup> of ØBHG1 were added to varying concentrations of both chemoheterotrophically and photosynthetically grown *Rp. blastica* (PMY) (section 2.3) and were incubated at 30°C. After 30 min the suspensions were titred for ØBHG1.

#### 2.6.4 The effect of cation concentrations on the rate of ØBHG1 adsorption

Chemoheterotrophically grown cells of *Rp. blastica* (PMY) were washed twice with 10 mM Tris-HCl (pH 6.8 in deionised water), then resuspended to a concentration of  $5 \times 10^8$  cells ml<sup>-1</sup> in the same buffer. The cell suspension was supplemented with the following: sodium chloride (NaCl), magnesium sulphate (MgSO<sub>4</sub>·7H<sub>2</sub>O), ammonium chloride (NH<sub>4</sub>Cl) or calcium chloride (CaCl<sub>2</sub>·2H<sub>2</sub>O) to final concentrations of 10 mM, 10 mM, 10 mM and 3 mM respectively.  $5 \times 10^6$  pfu ml<sup>-1</sup> of ØBHG1 were added and the rate of adsorption measured as described in section 2.6.2. The adsorption of



ØBHGl to a cell suspension in the Tris-HCl buffer, and PMY media were also monitored.

#### 2.6.5 Fluorescent labelling of cells

*Rp. blastica* was fluorescently labelled by the method of Hantke and Braun, (1974). Mid-exponential chemoheterotrophically grown *Rp. blastica* was washed twice in 0.9% (w/v) NaCl and adjusted to a concentration of  $1 \times 10^9$  cells  $\text{ml}^{-1}$  in this salt solution (section 2.3). To 5 ml of suspension, 50  $\mu\text{l}$  of  $6 \times 10^{-5}$  M 1-anilino-8-naphthalene sulphonate (ANS) were added. Throughout the protocol the cell suspension was maintained in the dark. After 15 min cells were pelleted by centrifugation and resuspended in the NaCl solution to  $5 \times 10^8$  cells  $\text{ml}^{-1}$ . ØBHGl was added to the cell suspension at increasing moles of 5, 10 and 20. Fluorescence of the samples were measured in a Perkin-Elmer fluorescence spectrophotometer at 35°C. Excitation and emission wavelengths were 360 nm and 450 nm respectively with a slit width of 1 cm.

#### 2.7 One-Step Growth Curves of ØBHGl on *Rp. blastica*

Two methods were employed for investigating the one-step growth curve of ØBHGl on both chemoheterotrophically and photosynthetically grown *Rp. blastica* (PMY).

##### 2.7.1 Method 1 - potassium cyanide

The growth curve of ØBHGl was followed by the method of Denhardt and Sinsheimer (1965) with the following modifications:

a) Chemoheterotrophic cultures

A mid-exponential culture of chemoheterotrophically grown *Rp. blastica* (PMY) was subcultured and grown to an OD of 0.16 ( $A_{650}$ ) corresponding to  $5 \times 10^8$  cells  $\text{ml}^{-1}$ . To this, potassium cyanide (KCN) buffered in 500 mM Tris HCl; pH 6.8, was added to a final concentration of 5 mM. The cell suspension was kept in the dark at  $30^\circ\text{C}$  for 20 min then ØBHG1 was added to give a moi of 0.02. After 50 min incubation, the suspension was diluted to  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  with prewarmed medium (PMY). At various time intervals samples were removed and titred for ØBHG1.

b) Photosynthetic cultures

The protocol was essentially identical to that above, with the following modifications. Throughout the procedure the cells were maintained under anaerobic conditions by fitting the sample flasks with a suba-seal and flushing with oxygen-free nitrogen for 5 min. Constant illumination at  $40 \text{ Em}^{-2} \text{ s}^{-1}$  was used. Samples were removed with a syringe and needle for titration of ØBHG1.

2.7.2 Method II - phage antisera

ØBHG1 was added to either chemoheterotrophically or photosynthetically grown *Rp. blastica* to a moi of 0.02. The phage/cell suspension was incubated at  $30^\circ\text{C}$  for 50 min. Crude antisera to ØBHG1 (section 2.8.1) were then added to a 50-fold dilution. After 5 min the cells were diluted to  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  in prewarmed medium and samples removed at various time intervals for titration of ØBHG1. Incubation conditions were as stated in Method I.

## 2.8 Preparation of Antisera

### 2.8.1 Antisera to ØBHG1

A rabbit was bled before being exposed to ØBHG1 to ensure that antibodies raised were specific to the phage, this was designated as the zero blood. The blood was stored overnight at 4°C and serum then separated from the blood clot by centrifugation at 2,000 g at 4°C for 15 min. The serum was stored in 1 ml aliquots at -20°C. 500 µl of  $10^{10}$  pfu ml<sup>-1</sup> caesium chloride purified ØBHG1 in PBS were emulsified with an equal volume of complete Freund's adjuvant by pumping in and out of a 2 ml glass hypodermic syringe until stiff. The procedure was repeated fortnightly for a period of six weeks before bleeding and then serum was collected as for the zero blood.

### 2.8.2 Antisera to whole cells of *Rp. blastica*

Chemoheterotrophically grown *Rp. blastica*, cultured on R8AH medium, was washed twice in sterile PBS and diluted to  $1 \times 10^6$  cells ml<sup>-1</sup>. 1 ml of this suspension was injected intravenously into the marginal ear vein of a New Zealand white rabbit. This was repeated weekly for a month. The inoculum was then increased to 1 ml of a  $1 \times 10^8$  cells ml<sup>-1</sup> cell suspension for a further two weeks after which the rabbit was bled and the sera collected as described in section 2.8.1.

## 2.9 Rocket Immunogel Electrophoresis

The presence of antibodies in sera was checked qualitatively by rocket gel immunoelectrophoresis. Immunoplates were prepared using 1% (w/v) agarose in barbitol buffer (50 mM sodium barbitol, 10 mM barbituric acid, pH 8.6) containing 0.5, 1 or 5% (v/v) crude antisera. Antigens were solubilised with a final concentration of 1% (w/v) Triton X-100.

20  $\mu$ l of sample was loaded into 2 mm diameter wells and electrophoresed at 8 mA for 16 hr at room temperature. Immunoplates were washed twice in PBS and once in double distilled water with partial drying between washes. Finally, the plates were dried in a hot air oven before being stained with coomassie blue for 10 min.

#### 2.10 Protein Immuno-Blotting

Antigens were detected by 10% (w/v) denaturing polyacrylamide gels (SDS PAGE) (section 2.20) and the "Western" immuno-blotting technique of Burnette (1981). After gel electrophoresis, the gel was placed onto the porous pad of a Bio-Rad Transblot cell soaked in transfer buffer (25 mM Tris-HCl; pH 8.3 and 0.192 M glycine dissolved in 20% (v/v) methanol). A nitrocellulose filter (Schleicher and Schuell, 0.45  $\mu$ m) was cut to the exact size of the gel and placed on top excluding all air bubbles. The gel-filter pad assembly was then placed into the cell which contained 3 L of transfer buffer and proteins were transferred from the gel (cathode side) to the filter (anode side) at 50 V (250 mA) for 3.5 hr at room temperature.

The procedure for the detection of protein antigens that interacted with the antisera was briefly as follows. The nitrocellulose filter was transferred into a series of solutions with continual gentle shaking at room temperature.

- i) 50 ml PBS containing 3% (w/v) bovine serum albumin (BSA) for 15 min.
- ii) 500  $\mu$ l of crude antisera was added and the filter left shaking for a further 60 min.

- iii) Five x 5 min washes in PBS.
- iv) 50 ml of 1% (w/v) BSA in PBS, and 125  $\mu$ l of biotinylated Protein A (Amersham International) for 1 hr.
- v) Five x 5 min washes in PBS containing 1% (w/v) Triton X-100.
- vi) 50 ml PBS containing 1% (w/v) BSA and 125  $\mu$ l of horseradish peroxidase (Amersham International) for 30 min.
- vii) Three x 5 min washes in PBS containing 1% (w/v) Triton X-100.
- viii) 50 ml PBS containing 0.025 g of 3,3'-diaminobenzidine (Sigma) and 0.015 g of nickel dichloride, to which 33  $\mu$ l of hydrogen peroxide (Amersham International) was added. Shaking was continued until the desired intensity of colour was obtained.
- ix) To stop developing the filter was transferred into PBS containing 1% (w/v) Triton X-100.

## 2.11 DNA Isolation and Restriction

### 2.11.1 $\phi$ BHG1 DNA isolation

DNA was isolated and purified by the method of Kirby (1967) with the following modifications. Phage were dialysed against lambda DNA buffer (0.01 M Tris, pH 8.0, 0.1 M KCl,  $10^{-4}$  M EDTA) overnight at 4°C. An equal volume of neutral phenol (equilibrated against 0.1 M Tris, pH 8.0) was added to the phage suspension. This was mixed gently for 20 min at 4°C, then phases were separated by centrifugation at 5,000 g for 1 min. The upper phase was removed and the lower phenol phase then re-extracted as before. To remove phenol the pooled aqueous phase was extracted 4 times with equal volumes of chloroform. DNA was precipitated twice with ethanol (2 vol of cold ethanol) then once with isopropanol (0.2 vol of 3 M Na acetate, pH 6.8, 0.54 vol isopropanol, stored overnight at -20°C). After drying, DNA was resuspended in a

minimal volume of TE and stored at  $-20^{\circ}\text{C}$ .

#### 2.11.2 Rn. blastic DNA isolation

Cells were harvested from batch cultures at 10,000 g,  $4^{\circ}\text{C}$  for 15 min. Cell pellets were washed in 0.05 volumes of TES buffer (100 mM Tris, 10 mM EDTA, 100 mM NaCl, pH 8 with conc. HCl) and resuspended in 12.5 ml of TES per g wet weight of cells. Lysozyme was added to a final concentration of  $1\text{ mg ml}^{-1}$  (Sigma Grade 1) and incubated at  $37^{\circ}\text{C}$  for one hour. Sodium lauryl sulphate (SLS) (2% (w/v) final concentration) was added and the cell suspension incubated at  $60^{\circ}\text{C}$  for 10 min with gentle occasional mixing. The cell lysate was made 1 M with respect to sodium perchlorate, and an equal volume of neutral phenol added. The suspension was mixed then spun for 15 min at  $4^{\circ}\text{C}$  to form two phases. The upper aqueous phase was removed then re-extracted with phenol and twice with phenol:chloroform:isoamylalcohol (25:24:1). The aqueous phase was then extracted with chloroform:isoamyl alcohol (24:1) until no white precipitate of proteins could be observed at the interface. NaCl (5 M) was added to the aqueous phase to a concentration of 0.25 M, followed by twice the volume of ice cold ethanol. The solution was mixed and left overnight at  $-20^{\circ}\text{C}$ . DNA was spun down at 5,000 g for 15 min at  $4^{\circ}\text{C}$  and the pellet dried under vacuum then resuspended in TE buffer, pH 8.0 (4 ml per g of original cell pellet). RNAase (pre-heat treated at  $90^{\circ}\text{C}$  for 10 min) was added to a concentration of  $100\text{ }\mu\text{g ml}^{-1}$  and the crude DNA incubated at  $37^{\circ}\text{C}$  for 30 min. This was then extracted twice with phenol:chloroform then repeatedly with chloroform:isoamyl alcohol until no white proteinaceous material appeared at the interface. The DNA was ethanol precipitated then finally resuspended in TE buffer and stored at  $-20^{\circ}\text{C}$ .

The concentration of DNA in the sample was calculated using the formula:

$$[\text{DNA}] \mu\text{g ml}^{-1} = \text{OD}_{260} \times 50 \times \text{dilution}$$

based on the assumption that DNA at a concentration of  $50 \mu\text{g ml}^{-1}$ , with a 1 cm light path has an absorbance value of 1.0.

#### 2.11.3 DNA restriction

Restrictions of DNA with endonucleases were performed as described by Maniatis *et al.* (1982). DNA fragments were separated on agarose gels (section 2.11.4) for analysis.

#### 2.11.4 Agarose gel electrophoresis

Samples of DNA were made up to 20  $\mu\text{l}$  with TE buffer, mixed with 5  $\mu\text{l}$  of gel loading buffer (GLB) and then run on 0.7% (w/v) agarose (Sigma, Type I) gels using TBE buffer.

Ethidium bromide ( $1 \mu\text{g ml}^{-1}$ ) was included in the gel and running buffer to allow DNA bands to be observed directly with a UV transilluminator after electrophoresis. Gels were photographed under UV light using a Polaroid camera.

##### TBE Gel Buffer 10x

108 g Trizma base

55 g Boric acid

made up to 1 L with double distilled water, pH 8.3

##### TBE Loading Buffer 5x

50% (v/v) glycerol

0.5% (w/v) bromophenol blue

#### 2.12 Determination of % G+C Content of DNA

The moles percent of guanine plus cytosine (% G+C) of ØBHG1 DNA was determined by the buoyant density method of Mandel *et al* (1968) in a Beckman Model E Analytical Ultracentrifuge. *Escherichia coli* K12 DNA (density of  $1.710 \text{ g cm}^{-3}$ ) was included as an internal standard.

#### 2.13 Preparation of Radiolabelled DNA by Nick Translation

A radioactive probe of ØBHG1 DNA was obtained by the method of Rigby *et al.* (1977). Labelled DNA was separated from unincorporated nucleotides after passage through a Sephadex G-50 column (5 ml volume) using gel filtration buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 50 mM NaCl). Radiolabelled DNA fractions were ethanol precipitated with  $30 \mu\text{g ml}^{-1}$  of added carrier DNA overnight at  $-70^{\circ}\text{C}$ . Precipitated fractions were spun for 25 min in a microfuge. Pellets were dried under a gentle stream of nitrogen then resuspended in the desired volume of  $0.1 \times \text{SSC}$ . Probes with a specific activity of  $>10^7 \text{ cpm } \mu\text{g}^{-1}$  of DNA were obtained using this method.

#### 2.14 Transfer of DNA from Agarose to Nitrocellulose Filters - Southern Blotting

DNA transfer from agarose gels to nitrocellulose filters was by the method of Southern (1975) with the following modifications. The gel was immersed in 0.5 M NaOH, 1.5 M NaCl for 40 min. (this resulted in denaturation of the DNA), and then 0.5 M Tris/HCl, pH 7.4, 3 M NaCl for a further 40 min. The gel was then placed onto a sheet of blotting paper soaked in  $20 \times$  saline sodium citrate (SSC) (3 M NaCl, 0.3 M sodium citrate) with its ends resting in a reservoir of  $20 \times \text{SSC}$ .



A nitrocellulose filter (Schleicher and Schuell, 0.45  $\mu$ m) cut to the exact dimensions of the gel, was soaked in 2 x SSC and layered on the bottom surface of the gel excluding any trapped air bubbles. A stack of blotting paper about 2 cm high was cut to the same dimensions as the gel and nitrocellulose. One sheet was soaked in 2 x SSC and placed on top of the nitrocellulose excluding all air bubbles, then the remaining paper added and the whole structure covered with 'cling-film'. A weight was placed on top and the transfer left overnight. The filter was then rinsed in 2 x SSC, blotted dry and baked in a vacuum oven at 80°C for 90 min.

#### 2.15 DNA-DNA Hybridisation Conditions

Because of the high G+C content of *Rp. blastica* DNA (69.4 mol %), 20% formamide (McConaughy et al., 1969) was included in the hybridisation solution to allow incubation at 55°C. Denhardt's solution (Denhardt, 1966) was used to prevent non-specific binding of probe DNA to the filter matrix.

Filters were placed in a polythene bag (Sterilin, Bibby UK) with 20 ml of prehybridisation solution (2 x SSC; 20% (v/v) formamide (deionised to pH 7 with Amberlite monobed resin 3B (Sigma); Denhardt's solution (0.02% (w/v) Ficoll 400, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin; 50  $\mu$ g ml<sup>-1</sup> denatured herring sperm DNA (Sigma)) then heat sealed to exclude all air bubbles. Sealed filters were incubated at 55°C for 2 hr. The prehybridisation fluid was then replaced with 6 ml of hybridisation fluid (= prehybridisation fluid + heat denatured probe (100°C for 5 min)), the filter resealed and incubated at 55°C for 26 hr. After hybridisation the filter was washed for three 20 min

periods in 2 x SSC at room temperature with gentle shaking then in 0.2 x SSC at 60°C for two 30 min periods. The filter was then dried over filter paper and autoradiographed as described in section 2.25.3.

## 2.16 Detection of Lysogeny in *Rp. blastica*

### 2.16.1 Induction with mitomycin C

Mitomycin C (Sigma) was added to flasks of early exponential chemoheterotrophically grown *Rp. blastica* and five cultures of spontaneously resistant *Rp. blastica* (section 2.4) to final concentrations of 1, 5 and 10  $\mu\text{g ml}^{-1}$ . Flasks were shaken for 2 days then checked for signs of lysis. Cells were pelleted and 20  $\mu\text{l}$  of the supernatants were spotted onto lawns of various members of the *Rhodospirillaceae* (Table 2).

### 2.16.2 Induction with ultra-violet radiation

#### a) Killing curve of *Rp. blastica*

A stationary phase culture of chemoheterotrophically grown *Rp. blastica* was washed once in sterile 0.01 M phosphate buffer; pH 6.8 then exposed to longwave ultra violet radiation at an intensity of 582  $\text{ergs sec}^{-1} \text{mm}^{-2}$ . At various times samples were removed and the number of viable cells determined by spreading dilutions onto PMY plates. The UV dosage required for 99% killing was used in the following:

#### b) Ultra-violet irradiation of cultures

1) A stationary phase culture of chemoheterotrophically grown *Rp. blastica* was exposed to ultra-violet radiation for 14 sec as described in 2.16.2a. The culture was pelleted, resuspended in PMY and incubated under standard chemoheterotrophic growth conditions. After 24 hr the

cells were pelleted and 20  $\mu$ l of supernatant spotted onto a range of members from the *Rhodospirillaceae* (Table 2).

ii) *Rp. blastica* strains resistant and sensitive to  $\phi$ BHG1 were patched onto nitrocellulose filters (Oxoid, 0.22  $\mu$ m) placed onto the surface of PMY plates and incubated overnight at 30°C. The filters were removed, and exposed to longwave UV radiation for 14 sec as described in 2.16.2a before being placed over lawns of *Rp. blastica*. Controls included  $\phi$ BHG1 streaked onto nitrocellulose filters and *Rp. blastica* unexposed to UV radiation.

#### 2.16.3 DNA-DNA hybridisation

2  $\mu$ g of unrestricted and EcoRI restricted  $\phi$ BHG1 DNA, and 2  $\mu$ g of Pst I restricted *E. coli*, *Rp. blastica* and DNA from three spontaneous resistant strains of *Rp. blastica* (section 2.4) were run on an agarose gel (section 2.11.4). This agarose gel was Southern blotted (section 2.14) and challenged with labelled  $\phi$ BHG1 DNA (section 2.13). The conditions for the DNA-DNA hybridisation were as given in section 2.15.

#### 2.17 Isolation of Peptidoglycan and the Outer Membrane and Cell Wall Fractions of *Rp. blastica*

##### 2.17.1 Method 1: Outer membrane isolation using gentle lysis

The method of Collins and Niederman (1976a) was followed for the isolation of outer membranes. Batch cultures of chemoheterotrophically grown *Rp. blastica* (PMY) were harvested and the pellet washed twice in 10 mM Tris-HCl, pH 8.1. The cells were resuspended in 25% (w/w) sucrose made in the Tris-HCl buffer and the following added at 10 min intervals to final concentrations as indicated: lysozyme (Sigma), 0.7 mg ml<sup>-1</sup>;

EDTA, 2.0 mg ml<sup>-1</sup>; Brij 58 (polyoxyethylene acetyl ether - Sigma), 0.65% (w/v); MgCl<sub>2</sub>, 11.5 mM, and a few crystals of deoxyribonuclease 1 (Sigma Grade 1). All additions were made at room temperature. Cell debris and whole cells were removed by sedimentation at 750 g for 10 min at 4°C.

Subcellular particles were isolated from the cell-free extracts by centrifugation for 60 min at 254,000 g in a Beckman SW 50.1 rotor. The pellets were resuspended in 10 mM HEPES buffer; pH 7.5, using a teflon homogeniser, then dialysed against the same buffer for 1 hr at 4°C. The homogenate was layered onto linear 35-50% (w/w) sucrose (in 10 mM HEPES, pH 7.5) gradients. These gradients were spun to equilibrium at 95,000 g for 13.5 hr in a Beckman SW28 rotor. Fractions were collected and the absorbance of these read at 235, 260 and 280 nm. 50 µl samples of these fractions were also analysed by SDS-PAGE (section 2.20).

#### 2.17.2 Method II - French Pressing

##### a) Isolation of cell envelopes and cell wall fractions

*Rp. blastica* and *Rp. blastica*<sup>r9</sup> were grown chemoheterotrophically on R8AH medium in a 12 L fermenter as described in section 2.1.3b. Cell envelopes and cell wall fractions were isolated according to the protocol of Flammann and Weckesser (1984a). Freshly harvested cells were resuspended in cold buffer (20 mM Tris-HCl, pH 8.0, 0.3 M sucrose, 2 mM EDTA) at approximately 3 ml per g of wet cells and frozen at -20°C for two to three days. Thawed cells were broken at 0°C by double passage through a French Pressure Cell (Amicon) at 110 kPa, DNAase and RNAase (Sigma Grade 1) added before the second passage. All the following steps were carried out at 4°C in 20 mM Tris HCl, pH 8.0. The homogenate was diluted with 3 volumes of cold buffer and centrifuged

twice at 7,500 g for 20 min. The supernatant was then centrifuged at 64,000 g in a Beckman 60 Ti rotor for 1 hr. The pellet representing the cell envelope fraction was homogenised in cold 20 mM Tris-HCl, pH 8.0, using a teflon homogeniser to a concentration of approximately 20 mg protein in 3 ml of buffer, then loaded onto a discontinuous sucrose density gradient. Gradients were modified to 55:43:35:19% (w/w) sucrose in the 20 mM Tris HCl buffer in the ratios of 5:4:9:4 ml respectively and were centrifuged at 113,000 g for 90 min in the above rotor. Each of the three bands were removed using a pasteur pipette and corresponding bands pooled. To remove sucrose from the fractions before passage down a second sucrose density gradient, the fractions were diluted with 2 volumes of 20 mM Tris HCl, pH 8.0. The top fraction was pelleted at 50,000 rpm (223,000 g) in a 75 Ti Beckman rotor while the lower two fractions were pelleted at 30,200 rpm (91,800 g) in a 60 Ti Beckman rotor for 1 hr. These pellets were resuspended and layered onto sucrose density gradients as above. Finally, the bands were removed and dialysed extensively against water at 4°C.

b) Triton X-100 extraction

Triton extraction of the lowest band was performed to remove any contaminating cytoplasmic membrane. Cell walls from the second sucrose density gradients were incubated in 2% (w/v) Triton X-100, 10 mM  $\text{MgCl}_2$ , 10 mM N-2-hydroxyethylpiperazine-N'-2-ethylsulfonic acid (HEPES) buffer, pH 7.4, at 23°C for 20 min at a protein concentration of 2 mg  $\text{ml}^{-1}$ . The purified cell walls were sedimented at 100,000 g for 1 hr in a Ti 60 Beckman rotor and washed twice with 10 mM  $\text{MgCl}_2$ , then five times with distilled water.

### 2.17.3 Isolation of peptidoglycan

Peptidoglycan was isolated from *Rp. blautica* by the procedure of Schmelzer *et al* (1982). 50 g wet weight of cells were washed in 20 mM Tris-HCl, pH 8.1, then broken by a double passage through a French Pressure Cell at 110 kPa. Whole cells were removed by centrifugation at 7,500 g for 20 min. The supernatant was ultracentrifuged at 64,000 g in a Beckman 60 Ti rotor at 4°C for 1 hr. The supernatant was discarded and the pellet of cell envelopes resuspended with a teflon homogeniser in a minimal volume of 20 mM Tris-HCl, pH 8.1 (approximately 5-10 ml). This homogenate was added drop wise into 300 ml of boiling water, containing 4% SDS (w/v) and 0.1%  $\beta$ -mercaptoethanol (v/v), for 15 min. This solution was allowed to cool then ultracentrifuged at 64,000 g in a Beckman 60 Ti rotor for 1 hr at 22°C. Centrifugation at 22°C was essential to prevent precipitation of SDS. The supernatant was discarded and the pellet re-extracted as above. After centrifugation the resulting pellet was washed with water approximately eight times to remove contaminating SDS. To test for SDS, 3 ml of chloroform and 0.5 ml of a 10% (w/v) methylene blue solution were added to approximately 1 ml of supernatant. If SDS was present methylene blue was found in the chloroform phase. Washing was continued until no blue colouration could be observed in the chloroform phase.

### 2.18 Lipopolysaccharide Extraction and Size Distribution Analysis

#### 2.18.1 Lipopolysaccharide extraction

Lipopolysaccharide was extracted from *Rp. blautica*, *Rp. blautica*<sup>9</sup> and *Rhodobacter sphaeroides* 8253 by two methods. The first was the phenol/water extraction procedure of Westphal *et al.* (1952), the second the phenol/chloroform/petroleum ether procedure of Galanos *et al.* (1969).

a) Method I - phenol/water

2 g of freeze dried *Rp. blastica*, *Rp. blastica*<sup>79</sup> or *Rb. sphaeroides* 8253 cells were resuspended in 100 ml of double distilled water at 67°C. 100 ml of 91% phenol (w/v), preheated to 67°C, were added and the mixture stirred continuously at 67°C for 20 min. The homogenate was cooled in iced water and was continuously stirred for 5 min. The homogenate was spun at 3,000 g at room temperature for 20 min. Centrifugation resulted in three distinct phases. The top aqueous phase was carefully removed before the lower two phases were reimmersed and heated to 67°C. 75 ml of double-distilled water, prewarmed to 67°C, were added to the homogenate, mixed, centrifuged and the phases separated as above. The water phases were pooled as were the remaining two phenol phases, and then were extensively dialysed against running water to remove phenol. Dialysis was over 2-3 days for the water phase and up to 1 week for the phenol phase. Cell debris was removed from the dialysed phenol phase by centrifugation at 2,000 g for 20 min. The volume of each phase was reduced by rotary evaporation at 22°C to approximately 30 ml. These concentrates were centrifuged at 105,000 g, 4°C for 4 hr in a 60 Ti Beckman rotor. The lipopolysaccharide pellet from each phase was resuspended in water with a teflon homogeniser and recentrifuged twice as above. Finally the supernatants from the centrifugation and the lipopolysaccharide pellets were freeze dried.

b) Method II - phenol/chloroform/petroleum/ether

*Rb. sphaeroides* 8253 was grown chemoheterotrophically. The cells were harvested and washed with 10 mM Tris-HCl, pH 8.1 before freeze drying. The extraction mixture contained liquid phenol (90 g phenol in 11 ml water), chloroform and petroleum ether in a volume ratio of 2:5:8 respectively. This mixture was clear if the phenol crystals were dry,

but if excess water was present in them the mixture was cloudy. This was clarified by adding solid phenol. 10 g of dried bacteria were placed in a glass beaker with 100 ml of extraction mixture and mixed for a few minutes. If the suspension became very viscous more extraction mixture was added. The homogenate was poured into a glass centrifuge vessel and debris removed by centrifugation at 3,000 g for 15 min. The supernatant which contained the crude lipopolysaccharide was filtered through Whatman No.1 filter paper into a round-bottom flask. The bacterial residue remaining was re-extracted once with the same amount of extraction mixture.

The supernatants were pooled and the petroleum ether and chloroform removed completely by brief rotary evaporation at 35°C. The solution was transferred to a glass centrifuge pot and water added dropwise, with mixing until all the lipopolysaccharide had precipitated and settled out. Care was taken not to add too much water as this would create two phases. The precipitated lipopolysaccharide was centrifuged at 3,000 g for 10 min, the supernatant decanted then the tube allowed to stand inverted and wiped with filter paper. The precipitate was washed three times with 5 ml of 80% phenol (w/v) and the tube wiped with filter paper as before. Finally, the precipitate was washed three times with ether to remove any residual phenol then dried *in vacuo*. The lipopolysaccharide was dissolved in 10 ml of distilled water, warmed to 45°C, then a vacuum applied carefully to remove air. This was shaken for a few minutes to obtain a viscous solution, then centrifuged at 23,100 rpm (100,000 g), 4°C for 4 hr in a Beckman SW40 rotor. The resulting colourless and transparent pellet, was dissolved in water and freeze-dried.



#### 2.18.2 Pronase treatment of purified lipopolysaccharide

5 mg (dry weight) of LPS (WPH) from *Rp. blastica* was resuspended in 2 ml of 0.01 M Tris-HCl, pH 6.8. Pronase (Boehringer) was added to a final concentration of  $2 \mu\text{g ml}^{-1}$ , and the solution incubated for 2 hr at  $30^{\circ}\text{C}$ . An equal volume of neutral phenol (section 2.11.1) was added, the mixture shaken well and dialysed overnight with running water to remove all traces of phenol before freeze drying.

#### 2.18.3 Sizing of lipid-free polysaccharide of *Rp. blastica*

10 mg of freeze dried LPS of *Rp. blastica* from the water phase (section 2.18.1) were resuspended in 100  $\mu\text{l}$  of 1% (v/v) glacial acetic acid and hydrolysed at  $100^{\circ}\text{C}$  for 30 min. The hydrolysate was microfuged for 3 min and the supernatant passed through a Millipore filter (MV - 0.45  $\mu\text{m}$ ). 20  $\mu\text{l}$  aliquots were applied to a Gilson high performance liquid chromatography (HPLC) system with a Rheodyne 7125 syringe injection valve and Knauer refractive index monitor under the following conditions:

Column:	TSK 4000 SEC
Column temperature:	ambient
Solvent system:	water
Flow rate:	$0.5 \text{ ml min}^{-1}$
Paper rate:	$0.15 \text{ cm min}^{-1}$
Integrator:	Shimadzu C-RIB chromatopac

## 2.19 Chemical Analysis of the Cell Envelope Components

### 2.19.1 Fatty acids

Fatty acids were analysed on a gas liquid chromatograph (GLC) after transesterification to their methyl ester derivatives. To 250  $\mu\text{g}$  of freeze-dried sample, 1 ml of 75% (v/v) methanol, 25% HCl (v/v) and 20  $\mu\text{l}$  of heptadeconic acid ( $1 \mu\text{g} \mu\text{l}^{-1}$  dissolved in benzol) as an internal standard, were added. The mixture was hydrolysed for 18 hr at  $100^{\circ}\text{C}$  then the volume reduced to approximately 500  $\mu\text{l}$  with a stream of nitrogen. 500  $\mu\text{l}$  of half-saturated sodium chloride were added and the contents well mixed. 1 ml of chloroform was added, the solutions mixed, then the upper chloroform phase was carefully removed to prevent contamination from the lower phase. This extraction was repeated five times. The chloroform phases were pooled and the extract dried with a stream of nitrogen. The extract was resuspended in 10  $\mu\text{l}$  of chloroform and 1  $\mu\text{l}$  was injected into the GLC under the following conditions:

Gas chromatograph:	Varian Aerograph, series 14000 with flame ionizer detector (FID)
Column:	glass column (150 cm x 0.3 cm)
Column packing:	15% EGSS-X with Gaschrom P, 100-200 mesh
Temperature:	column - $240^{\circ}\text{C}$ detector - $240^{\circ}\text{C}$ injector - $240^{\circ}\text{C}$
Gas:	carrier gas - $\text{N}_2$ (30 ml $\text{min}^{-1}$ ); gas for FID - $\text{H}_2$ (30 ml $\text{min}^{-1}$ ), Air (300 ml $\text{min}^{-1}$ )
Plotter:	Varian A-25
Paper rate:	0.5 cm $\text{min}^{-1}$
Integrator:	Varian Aerograph, Model 485

## 2.19.2 Neutral sugar

### a) Thin-layer chromatography

To 1 mg of freeze-dried lipopolysaccharide, 100  $\mu$ l of 0.5 M  $\text{H}_2\text{SO}_4$  was added the contents mixed then hydrolysed for 4 hr at 100°C. The hydrolysate was neutralized with a saturated solution of barium hydroxide. Small samples were removed and spotted onto indicator paper to test for neutralisation. The hydrolysis tubes were then spun at 3000 g for 15 min to pellet barium sulphate formed during neutralisation. The supernatants were carefully removed and freeze dried. 10  $\mu$ l of water was added to resuspend the hydrolysates. Samples were then loaded onto cellulose coated glass plates (Merck) by multiply spotting 2  $\mu$ l aliquots and drying between each application. 3  $\mu$ l of sugar standards were also loaded (10 mg  $\text{ml}^{-1}$  of glucose, rhamnose, mannose, galactose, ribose and fucose).

Plates were placed into a tank containing a solvent system of n-butanol:pyridine:water in the ratio of 6:4:3 vols. The solvent had been stored in the tank for 24 hr to allow the atmosphere to become saturated. The plates were run until the solvent front was approximately 1 cm from the top (4 hr). The plates were then removed, allowed to dry, then returned to the tank and re-run in the same direction. This was found to give a better separation of the sugars. After drying, the plates were stained by spraying with anilinium hydrogen phthalate (0.93 g aniline, 1.6 g O-phthalic acid in 100 ml of n-butanol saturated with water). Sugars could be observed after baking at 100°C for 10-15 min.

b) Gas-liquid chromatography

Neutral sugars were quantified on a GLC after synthesizing their alditol acetate derivatives. 1 mg of freeze-dried sample was hydrolysed for 48 hr at 100°C with 1 M HCl. To this 20  $\mu$ l of inositol (1  $\mu$ g  $\mu$ l<sup>-1</sup>) was added as an internal standard. Samples were neutralised with Amberlite exchange resin (Amberlite AG type IRA-410). The resin was activated by soaking in a saturated solution of sodium bicarbonate overnight and then washing with water until at pH 7. The hydrolysate was added to a tube containing a small amount of the resin, mixed well, centrifuged for 2 min in a microfuge then the supernatant removed. The resin was then washed a further four times with 700  $\mu$ l of water. The pooled supernatants were rotary evaporated at the standard temperature of 25°C. When dry, 1 ml of 0.4% (w/v) sodium hydrogen borate was added, and left to stand overnight at room temperature. Sodium ions were removed by addition of a cation exchanger (Dowex 50 WX-8 (H<sup>+</sup> form)), using the same procedure as for the Amberlite resin. The pooled supernatants were dried by rotary evaporation. To destroy the hydrogen borate ions, 250  $\mu$ l of 2 N acetic acid were added and then removed by rotary evaporation. When dry, five x 1 ml methanol washes were performed, the methanol being removed by rotary evaporation. 0.5 ml of acetic anhydride:pyridine in the ratio of 1:1 (v/v) was added to the tube which was sealed and heated to 100°C for 30 min. The tube was then washed with 1 ml aliquots of water until the sample did not smell of pyridine. The sample was then dried and three 300  $\mu$ l aliquots of chloroform added to remove the sugar derivatives. The chloroform was placed into conical glass tubes and dried. 10  $\mu$ l of chloroform was added, and 1  $\mu$ l was injected into the GLC under the following conditions:

Gas chromatograph:	Varian Aerograph, series 14000 with flame ionizer detector (FID)
Column:	glass column (150 cm x 0.3 cm)
Column packing:	3% ECNSS-X with Gaschrom Q, 100-200 mesh
Temperature:	column - 180°C detector - 240°C injector - 240°C
Gas:	carrier gas - N <sub>2</sub> (30 ml min) <sup>-1</sup> ; gas for FID - H <sub>2</sub> (30 ml min) <sup>-1</sup> , Air (300 ml min) <sup>-1</sup>
Plotter:	Varian A-25
Paper rate:	0.5 cm min <sup>-1</sup>
Integrator:	Varian Aerograph, Model 485

c) High performance liquid chromatography

Samples weighing greater than 5 mg were resuspended in 1% (v/v) glacial acetic acid and hydrolysed for 60 min at 100°C. Hydrolysates were then centrifuged at 5000 g for 15 min and the supernatants extensively dialysed against deionized water followed by freeze drying. 400 µl of 0.4 N H<sub>2</sub>SO<sub>4</sub> were then added and the samples hydrolysed at 100°C for 16 hr. Amberlite IR410 resin in the bicarbonate form was added to neutralise the samples. These solutions were rotary evaporated to dryness at 50°C, dissolved in approximately 200 µl of water, microfuged for 3 min and passed through Millipore filters (HV 0 0.45 µm). Samples weighing less than 5 mg were hydrolysed immediately with 0.4 N H<sub>2</sub>SO<sub>4</sub> and treated as above. 20 µl aliquots were then applied to a Gilson HPLC system under the following conditions:

Column: Brownlea Pb<sup>2+</sup>  
Column temperature: 85°C  
Solvent system: water  
Flow rate: 0.2 ml min<sup>-1</sup>  
Paper rate: 0.2 cm min<sup>-1</sup>  
Integrator: Shimadzu C-RIB chromatopac

### 2.19.3 Amino acid analysis

Amino acids were measured on a Biotronik-Amino acid analyser LC 6001 with an automatic sample inject system (sample injector BT 7040). The main wave-lengths were at 570 nm, and 440 nm for proline analysis. 200-250 µg of freeze-dried material was hydrolysed at 105°C for 18 hr with 400 µl of 4 N HCl. The hydrochloric acid was removed with nitrogen at 37°C. 200 µl of sample buffer was then added and from this 50 µl were injected into the amino acid analyser. The concentration of the individual amino acids were determined by the intensity of the ninhydrin reaction and automatically recorded.

#### Buffers and Solutions:

- a) Ninhydrin solution: per litre
- |        |                                |
|--------|--------------------------------|
| 750 ml | Ethylenglycolmonoethylether    |
| 250 ml | 4M Na acetate buffer, pH 5.5   |
| 20 g   | Ninhydrin                      |
| 10 ml  | Titanium-III chloride solution |

b) Sample buffer:

The composition of the sample and elution buffer is given in Tables 3 and 4 A-E. The composition of the column regeneration solution is given as buffer F.

Table 3.

Programme Steps: Analysis contained the following programme steps:

Programme for the Amino Acid Analyser

Programme	Buffer	Column temp.	Time in min	Notes
1	A	T <sub>1</sub>	5	Before injection
2	A	T <sub>1</sub>	13	After injection
3	B	T <sub>1</sub>	10	
4	B	T <sub>2</sub>	10	
5	C	T <sub>2</sub>	14	
6	D	T <sub>2</sub>	30	
7	E	T <sub>2</sub>	35	
8	F*	T <sub>3</sub>	6	Regeneration
9	A	T <sub>2</sub>	10	
10	A	T <sub>2</sub>	3	
11	A	T <sub>2</sub>	10	

\* - Regeneration solution

Analysis Conditions:

Pre-wash column : packing material: Type BTC F  
 liquid height: 40 mm

Separating column : packing material: Type BTC 2710  
 liquid height: 207 mm

Buffer flow rate : 20 ml h<sup>-1</sup>

Ninhydrin flow rate : 20 ml h<sup>-1</sup>

Column temperature : T<sub>1</sub> = 50°C  
 T<sub>2</sub> = 58°C  
 T<sub>3</sub> = 63°C

Integrator : Shimadzu C-R 1B

Paper Rate : 2 mm/min

Table 4. Composition of buffers required for amino acid analysis

	Buffer A	Buffer B	Buffer C	Buffer D	Buffer E	Regeneration solution	Sample buffer
Final pH	3,31	3,87	4,50	6,00	10,55	-	2,20
Sodium - normalities	0,10 N	0,10 N	0,10 N	0,18 N	0,16 N	0,30 N	0,10 N
Citrate - molarity	0,10 M	0,10 M	0,08 M	0,06 M	0,03 M	-	0,10 M
tri-sodium citrate x 2H <sub>2</sub> O	9,8 g	9,8 g	9,8 g	17,6 g	-	-	9,8 g
citric acid x H <sub>2</sub> O	14,0 g	14,0 g	9,8 g	-	6,9 g	-	14,0 g
Methylcellulose	45,0 ml	-	-	-	-	-	-
Boric acid	1,0 g	1,0 g	1,0 g	1,0 g	5,0 g	-	-
Hydrochloric acid 37%	1,0 ml	*	*	3,0 ml	-	-	1,0 ml
Sodium hydroxide	-	*	*	-	5,0 g	12,0 g	-
Brij 35 (25% soltn. in H <sub>2</sub> O)	1,0 ml	1,0 ml	1,0 ml	1,0 ml	1,0 ml	1,0 ml	-
2,2'-Thiodiethanol (25%)	-	-	-	-	-	-	20,0 ml
END Volume	1,0 l	1,0 l	1,0 l	1,0 l	1,0 l	1,0 l	1,0 l



#### 2.19.4 Determination of protein concentration

Protein concentrations were determined with the Folin-phenol reagent using crystalline bovine serum albumin as a standard (Lowry et al., 1951). Briefly the method was as follows:

##### Solutions were:

- A 5% (w/v) sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) solution
- B 0.5% (w/v) cupric sulphate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ), 1% (w/v) sodium potassium tartrate [ $\text{NaK}(\text{C}_4\text{H}_4\text{O}_6)_2$ ]
- C 2 ml of B was added to 50 ml of A (made fresh daily)
- D Folin-Ciocalteu phenol reagent diluted to 0.1 N acidity
- E 1 N sodium hydroxide ( $\text{NaOH}$ ) solution

##### Procedure:

To the sample was added 0.5 ml of 1 N sodium hydroxide and 2.5 ml of solution C. It was then allowed to stand for 10 min. Solution D (0.5 ml) was then added and allowed to stand for 30 min. The optical density was then measured against a reagent blank with distilled water at 650 nm in 1 cm cuvettes.

#### 2.20 Gel Electrophoresis of Proteins

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a discontinuous buffer system (Laemmli, 1970) including the ionic detergent sodium dodecyl sulphate (SDS) to dissociate proteins into their individual polypeptide subunits.

### 2.20.1 Solutions

Gels were cast between glass plates (20 x 25 cm) with a stacking gel polymerised on top of the resolving gel. Glass plates were separated by lucite spacers with edges sealed by polypropylene tubing smeared with vaseline, and held together with bulldog clips.

The following gel solutions were used:

<u>30% (w/v) Acrylamide mixture</u>	<u>20 ml</u>
60% (w/v) low bisacrylamide	10 ml
75% (v/v) glycerol	7.3 ml
lower gel buffer	2.5 ml
10% (w/v) SDS	0.2 ml

Degassed for 5 mins in a vacuum desiccator before 4  $\mu$ l of TEMED (NNN'-Tetra-methylethylenediamine) and 40  $\mu$ l of freshly prepared ammonium persulphate (APS) (10% w/v) were added.

<u>10% (w/v) Acrylamide mixture</u>	<u>75 ml</u>
60% (w/v) high bisacrylamide	12.5 ml
H <sub>2</sub> O	52.3 ml
lower gel buffer	9.4 ml
10% (w/v) SDS	0.75 ml

Degassed for 5 min before 15  $\mu$ l of TEMED and 150  $\mu$ l of freshly prepared (10% w/v) APS were added.

<u>Stacking gel mixture</u>	<u>10 ml</u>
stacking gel acrylamide	3.0 ml
H <sub>2</sub> O	4.4 ml
stacking gel buffer	2.4 ml
10% (w/v) SDS	0.1 ml

Degassed for 5 min before 5  $\mu$ l of TEMED and 100  $\mu$ l of freshly prepared APS were added.

#### 40% (w/v) Acrylamide stocks

##### Low % gel (high bis)

60 g	acrylamide (Eastman Kodak)
1.6 g	bisacrylamide (Eastman Kodak)

##### High % gel (low bis)

60 g	acrylamide
0.3 g	bisacrylamide

Both made up to 100 ml with double distilled water

#### Stacking gel acrylamide

10 g	acrylamide	made up to 100 ml with
0.5 g	bisacrylamide	double-distilled water

#### Buffers

##### Lower gel buffer

36.33 g	Trizma base (3 M)	made up to 100 ml with
conc. HCl	to give pH 6.8	double distilled water (pH 6.8)

#### Stacking gel buffer

5.98 g	Trizma base (0.05 M)	made up to 100 ml with
conc. HCl	to give pH 6.8	double distilled water (pH 6.8)

#### Running buffer (5xstock)

30.2 g	Trizma base	in 1 litre of double distilled
144 g	glycine	water

Running buffer - 200 ml of stock + 10 ml (w/v) SDS per litre.

#### Sample buffer (4xstock)

2 x conc. stacking gel buffer	125 $\mu$ l
glycerol	25 $\mu$ l
20% (w/v) SDS	200 $\mu$ l
2- $\beta$ -mercaptoethanol	100 $\mu$ l
0.5% (w/v) bromophenol blue	50 $\mu$ l

#### 2.20.2 Gel formation

Two types of gels were used to resolve soluble proteins, a 10-30% (w/v) concave gradient gel and more often a 10% (w/v) linear gel.

The pouring of a concave 10-30% (w/v) gradient gel of 75 ml total volume required 20 ml of a 30% (w/v) acrylamide mixture placed in a mixing chamber. 50 ml of 10% (w/v) acrylamide was pumped into the mixing chamber using a peristaltic pump and thoroughly mixed with the 30% (w/v) acrylamide solution. The volume of the liquid in the mixing chamber remained constant and the gel was poured with the 30% (w/v) acrylamide constantly being diluted by the 10% (w/v) solution.

10% (w/v) linear gels were prepared by pumping a single 10% (w/v) acrylamide solution of 75 ml through a mixing chamber and between the glass plates.

After pouring the gel mixture was overlaid with water saturated butanol and allowed to polymerise. When set, the butanol was washed off with double distilled water and a stacking gel poured above the resolving gel with a lucite slot former pushed into the top of the stacking gel. After setting, the polypropylene tubing and slot former were removed from the gel before being placed in an electrophoresis tank for loading. Care was taken that on addition of the running buffer to the tank no air bubbles were trapped between the plates below the gel. Any such air bubbles were removed with a 10 ml syringe and hooked needle filled with running buffer.

#### 2.20.3 Sample preparation

The amount of protein sample loaded on a gel varied depending on the protein concentration of the sample and the method of gel staining used. For a gel stained by coomassie blue, 50-110  $\mu$ g of soluble protein was loaded per gel slot, while a gel stained with silver nitrate required 5-10  $\mu$ g of soluble protein. Samples were denatured by adding 0.33 vol of 4 x sample buffer and boiling for 5 min. Denatured samples were loaded into the individual gel slots using a Hamilton 0.1 ml glass syringe, which was thoroughly rinsed with double distilled water between samples.

#### 2.20.4 Electrophoresis conditions

Electrophoresis was carried out at 4°C at 12 mA for 10-30% (w/v) acrylamide gels and 8 mA for a 10% (w/v) linear gel, until the bromophenol blue dye front reached the bottom of the gel. Gels were

then removed from between the plates, fixed and stained accordingly.

2.20.5 7.5% (w/v) linear semi-denaturing polyacrylamide gel  
electrophoresis

This method was developed to retain maximal activity and minimal denaturation of outer membrane proteins, but still, however, allowing some electrophoretic separation. The method of Alderman *et al.* (1979) was followed but modified to run cell wall proteins on the slab gel system.

2.21 Gel Staining

2.21.1 Coomassie blue stain

Gels were immersed in the staining solution (45% (v/v) methanol, 10% (v/v) glacial acetic acid and 0.1% (w/v) coomassie blue R250) for 3-4 hr and then destained in 45% (v/v) methanol, 10% (v/v) glacial acetic acid until background colouration was at a minimum.

2.21.2 Silver stain

Silver stain, being approximately 10-20 times more sensitive than coomassie blue, allowed the observation of protein bands which otherwise could remain undetected. The procedure of Wray *et al.* (1981) was used. Briefly the method was as follows:

- i) The gel was soaked in 50% (v/v) methanol for at least 6 hr with a total of three changes.
- ii) The staining solution was as follows:  
Solution A - 1.6 g of silver nitrate dissolved in 8 ml of double distilled water.

Solution B = 52 ml of 0.36% (w/v) sodium hydroxide solution  
with 2.5 ml of 14.8 M ammonium hydroxide  
solution.

Solution A was added to solution B with constant mixing and  
finally made up to 200 ml with double distilled water.

- iii) The gel was soaked in the stain for 20 min.
- iv) The gel was then washed in deionised water for two 5 min  
periods.
- v) Developing solution: 2.5 ml of 1% (w/v) citric acid and 0.45 ml  
of 37% (w/v) formaldehyde solution made up to 500 ml with  
deionised water. The gel was soaked in developing solution  
until bands appeared - approximately 10-15 min.
- vi) Development was stopped by soaking the gel in 50% (v/v)  
methanol, 5% (v/v) acetic acid.

Gels were photographed using Kodak Panatomic-X 35 mm film (32 ASA) on a  
fluorescent light transilluminator.

## 2.22 Enzyme Assays

All enzyme assays were performed at 25°C in 3 ml reaction volumes using  
1 cm lightpath quartz cuvettes in a Fye Unicam SP8 double beam  
spectrophotometer with an integral chart recorder.

### 2.22.1 Succinate dehydrogenase

Succinate dehydrogenase was assayed by the phenazine methosulphate (PMS)  
mediated succinate dependent reduction of DCPIP at 600 nm in a modified  
version of that described by King (1963). The reaction contained ( $\mu$ mol  
in 3 ml): potassium phosphate buffer, pH 7.6, 150; DCPIP, 0.2; PMS,

1.2; Na succinate, 20; K cyanide, 7.0. The blank lacked DCPIP. The reaction mixture was prepared without succinate, the appropriate sub-cellular fraction added and then pre-incubated at 25°C for 2 min before the succinate was added. The initial rate of absorbance decrease was recorded.

#### 2.22.2 NADH and NADPH oxidase systems

The NADH and NADPH oxidase systems were assayed by measuring the decrease in absorbance at 340 nm after the addition of the appropriate sub-cellular fraction to a mixture containing ( $\mu\text{mol}$  in 3 ml) (Oelze and Kamen, 1975):

Tris-HCl pH 7.4	200
NADH or NADPH	0.6

The blank lacked NADH.

#### 2.23 Electron Microscopy

Samples were examined after negative staining with phosphotungstic acid in the following manner. A drop of suspension was placed on Formvar-coated copper grids (Agar Aids, 100 segment mesh; 3.05 mm diam) for 30 sec-1 min then excess liquid was removed with a strip of filter paper. The grid was allowed to dry and the sample negatively stained by placing a drop of 1% (w/v) phosphotungstic acid (pH 7.0) onto the grid and immediately removing it with a strip of filter paper. Specimens were examined using a Joel JEM-100S transmission electron microscope operating at an accelerating voltage of 80 kV. Photographs were taken using Kodak 4489 Ester thick base electron microscope film which was developed in Kodak D-19 developer and fixed in Kodafix fixer according to the manufacturers instructions.



2.24 Determination of the Cell Envelope Components Involved in  $\phi$ BHG1 Adsorption

2.24.1  $\phi$ BHG1 adsorption to cell wall fractions

The rates of adsorption of  $10^3$  pfu  $\text{ml}^{-1}$   $\phi$ BHG1 to 150  $\mu\text{g}$  of protein from the two cell wall fractions (B1 and B2) isolated in section 2.17.2, from both *Rp. blastica* and *Rp. blastica*<sup>r9</sup> were followed as described in section 2.6.2. The concentration of protein was determined as in section 2.19.4.

2.24.2 Effect of pre-heat treatment of *Rp. blastica* on  $\phi$ BHG1 adsorption

Mid-exponential chemoheterotrophically grown *Rp. blastica*, was pelleted and washed twice in 10 mM Tris-HCl, pH 7.3. The cells were resuspended to a concentration of  $5 \times 10^8$  cells  $\text{ml}^{-1}$ . 1 ml aliquots of the cell suspension were heated to 30°C, 40°C or 60°C for 1 hr and then 30°C for 5 min. 10  $\mu\text{l}$  of  $\phi$ BHG1 ( $10^5$  pfu  $\text{ml}^{-1}$ ) was added  $\text{ml}^{-1}$  and the rate of adsorption followed as described in section 2.6.2.

2.24.3 Effect of pronase digestion of *Rp. blastica* on  $\phi$ BHG1 adsorption

Mid-exponential chemoheterotrophically grown *Rp. blastica* was pelleted and washed twice in 10 mM Tris-HCl, pH 7.3. The cell concentration was adjusted to  $5 \times 10^7$  cells  $\text{ml}^{-1}$  in 10 mM Tris-HCl, pH 7.3 (section 2.3) and pronase (Boehringer) added to a concentration of 250  $\mu\text{g}$   $\text{ml}^{-1}$ . The cells were incubated at 30°C for 30 min then washed twice in the Tris-HCl buffer and resuspended to a concentration of  $5 \times 10^8$  cells  $\text{ml}^{-1}$  in the same buffer. 10  $\mu\text{l}$  of  $\phi$ BHG1 ( $10^5$  pfu  $\text{ml}^{-1}$ ) were added  $\text{ml}^{-1}$  and the rate of adsorption followed (section 2.6.2). Traces of active pronase that remained on the cell surface after washing and dilution were assumed to be less than 2  $\mu\text{g}$   $\text{ml}^{-1}$ . The stability of  $\phi$ BHG1 in the presence of 2  $\mu\text{g}$   $\text{ml}^{-1}$  of pronase was tested by adding  $10^5$  pfu  $\text{ml}^{-1}$  of

ØBHCl to 10 mM Tris-HCl, pH 7.3 containing  $2 \mu\text{g ml}^{-1}$  of pronase and titrating the suspension after 1 hr incubation at  $30^{\circ}\text{C}$ .

#### 2.24.4 ØBHCl adsorption to lipopolysaccharide

The rate of adsorption of ØBHCl to 1 mg dry weight of LPS extracted from *Rp. blastica* and *Rp. blastica*<sup>r<sub>9</sub></sup> was measured as described in section 2.16.2. Two methods were employed to resuspend the freeze dried LPS, homogenisation with a teflon homogeniser or two 30 sec pulses of sonication (12 microns peak to peak), with cooling on ice for 1 min between pulses.

#### 2.25 Detection of ØBHCl Encoded Protein Kinase

##### 2.25.1 Labelling of infected cells

The method of Hodgson *et al.* (1985) was followed. A mid-exponential chemoheterotrophically grown culture of *Rp. blastica* was sub-cultured and 25 ml grown until an OD of 0.16 (650 nm), corresponding to  $5 \times 10^8$  cells  $\text{ml}^{-1}$  was reached. 5 mCi of  $^{32}\text{P}$ -orthoadenosine triphosphate (Amersham) and ØBHCl to a moi of 10 were added. At 20 min intervals 5 ml of phage/cell suspension were removed and added to a cooled glass universal containing 100  $\mu\text{l}$  of 2 M sodium azide. The cells were then pelleted by centrifugation at 5,000 g,  $4^{\circ}\text{C}$  for 15 min and washed twice in cold 20 mM Tris-HCl, pH 7.5 containing 40 mM  $\text{NaN}_3$ . The pellet was resuspended in a minimal volume of Tris-HCl buffer and freeze dried. Samples were then resuspended in 30  $\mu\text{l}$  of water and analysed by SDS-PAGE (section 2.20).

#### 2.25.2 Trichloroacetic acid precipitation of proteins

After SDS-PAGE the proteins were TCA precipitated by a modification of the method of Bhorjee and Perderon (1976). The gel was placed into a glass dish containing 150 ml of 80% (w/v) TCA and 600 ml double distilled water. This dish was then lowered into a pre-heated water bath at 85°C for 40 min. The gel was then cooled and washed for two 2.5 hr and one 12 hr periods in a solution of 200 ml of 50 (w/v) TCA and 500 mM disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ); 700 ml methanol; 1.1 L of double distilled water.

#### 2.25.3 Autoradiography

Prior to autoradiography gels were dried at 80°C under vacuum. The dried gel was placed in contact with X-ray film (Kodak No-screen) in a sealed case and stored at -75°C. The film was developed after 12 and 24 hr. Films were developed in Kodak DX-80 developer and fixed in Kodak FX-40 fixer.

#### 2.26 Detection of a $\phi$ BHGI Encoded Lipopolysaccharide Hydrolase

To 1 ml of  $1 \times 10^{10}$  pfu  $\text{ml}^{-1}$   $\phi$ BHGI suspended in deionized water, pH 7, 10  $\mu\text{l}$  of a 1.15 mg  $\text{ml}^{-1}$  solution of *Rp. blastica* LPS (WPH), were added. The suspension was mixed, incubated at 30°C for 24 hr and then freeze dried. Controls of 1 ml of  $1 \times 10^{10}$  pfu  $\text{ml}^{-1}$  of  $\phi$ BHGI and 10  $\mu\text{l}$  of 1.15 mg  $\text{ml}^{-1}$  *Rp. blastica* LPS (WPH) in 1 ml of deionized water, pH 7, were also included. Following freeze drying, samples were resuspended in 10  $\mu\text{l}$  of double distilled water, pH 7, and tested for the presence of free sugars by running on cellulose thin layer chromatography plates (section 2.19.2a).

## 2.27 RNA Polymerase Isolation

RNA polymerase was isolated using column affinity chromatography.

### 2.27.1 Cell lysis

500 ml of *Rp. blasatica* were grown chemoheterotrophically to an OD of 0.16 ( $A_{650}$ ) corresponding to  $5 \times 10^8$  cells  $\text{ml}^{-1}$ . The cells were pelleted at 10,000 g, washed in 10 mM Tris-HCl, pH 8.0 and resuspended in 1.5 ml lysis buffer containing 50 mM NaCl before freezing at  $-20^\circ\text{C}$ . This cell suspension was freeze-thawed twice, and then 25  $\mu\text{l}$  of 5% (w/v) sodium deoxycholate were added, the mixture being kept on ice. After 20 min, 300  $\mu\text{g}$  of lysozyme were added, with gentle mixing, and left on ice for a further 40 min. Cells were broken by 30 sec pulses of sonication at 12 microns peak to peak with cooling on ice between each period. Cell debris was removed by centrifugation at 10,000 g for 40 min at  $4^\circ\text{C}$ .

<u>lysis buffer x10</u>	<u>g l<sup>-1</sup></u>	<u>lysis buffer x1</u>	<u>10 ml</u>
100 mM Tris-HCl, pH 8.0	1.21	lysis buffer x10	1.0 ml
20 mM EDTA	0.74	0.5 M dithiothreitol	6 $\mu\text{l}$
		50% (w/v) glycerol	1.5 ml
		water	7.49 ml

### 2.27.2 Column chromatography

#### a) Method 1

A heparin-agarose (Sigma) column was packed as follows: a slurry of the agarose was packed to a height of 2.5 ml in a 10 ml plastic syringe plugged with a small amount of glass wool, and then washed with 50 ml of heparin-agarose buffer (HAA) containing 50 mM NaCl at the standard temperature of  $4^\circ\text{C}$ . The cellular extract was loaded onto the column then allowed to sit for at least 1 hr. The column was washed with 30 ml

of HAA buffer containing 50 mM NaCl and 2 ml fractions collected using a LKB Gamma Fraction Collector. The second wash was a 30 ml linear gradient of HAA buffer containing 50 mM to 1.2 M NaCl, collecting 1 ml fractions. 50  $\mu$ l samples from all these fractions were analysed by SDS-PAGE (section 2.20). Gradient fractions containing RNA polymerase, as determined by the SDS-PAGE, were pooled and dialysed against 1 L of buffer C containing 50 mM NaCl for approximately 4 hr.

Double stranded DNA-cellulose (ds-DNA cellulose) (Sigma) was hydrated overnight in Buffer C containing 0.1 M NaCl. A 3.5 ml column was packed as above, loaded with the dialysed RNA polymerase fractions then washed with 50 ml of Buffer C containing 0.1 M NaCl. The second wash was a 30 ml gradient of Buffer C containing 0.1 to 1.4 M NaCl and the third 5 ml of Buffer C containing 4 M NaCl. 1 ml fractions were collected for all three washes.

Fractions collected from the salt gradient and 4 M salt wash were dialysed against 1 L of 10 mM Tris-HCl, pH 7.0, freeze-dried and analysed by SDS-PAGE.

#### Solutions

<u>HAA Buffer x10</u>	<u>g l<sup>-1</sup> of distilled water</u>
100 mM Tris-HCl, pH 8.0	6.05
100 mM MgCl <sub>2</sub> ·6H <sub>2</sub> O	10.16
10 mM EDTA	1.86

<u>HAA Buffer x1</u>	<u>100 ml</u>
10x HAA buffer	10 ml
0.5 M dithiothreitol (DTT)	60 $\mu$ l
50% (w/v) glycerol	15 ml
water	74.94 ml

<u>Buffer C x10</u>	<u>g<sup>-1</sup> of distilled water</u>
0.5 M Tris-HCl, pH 8.0	60.55
0.001 M EDTA	0.29
0.1 M MgCl <sub>2</sub> .6H <sub>2</sub> O	20.33

b) Method II

The cellular extract was loaded directly onto the ds DNA cellulose column and allowed to sit for at least 1 hr before the column was washed as described above.

2.27.3 Isolation of RNA polymerase from  $\phi$ BHG1 infected cells

$\phi$ BHG1 was added to a moi of 10 to 500 ml of a  $5 \times 10^8$  cells ml<sup>-1</sup> chemoheterotrophically grown *Rp. blastica* culture. After 50 min incubation at 30°C, sodium azide was added to a final concentration of 10 mM. The cells were pelleted at 10,000 g, 4°C, and washed in 10 mM Tris-HCl, pH 8.0. RNA polymerase was isolated as described in sections 2.27.1 and 2.27.2b.

## 2.28 Transduction

### 2.28.1 Isolation of spontaneous kanamycin resistant mutants of

#### *Rp. blastica*

$10^9$  cells of chemoheterotrophically grown *Rp. blastica* were spread onto the surface of PMY plates containing  $50 \mu\text{g ml}^{-1}$  of kanamycin (Km). After 48 hr, colonies were picked and restreaked onto kanamycin plates to confirm resistance.

### 2.28.2 Preparation of phage

$5 \times 10^8$  cells of kanamycin resistant *Rp. blastica* grown chemoheterotrophically on PMY containing  $50 \mu\text{g ml}^{-1}$  kanamycin were infected at an moi of 0.01 with ØBHG1. Progeny ØBHG1 were purified as indicated in section 2.2.2.

### 2.28.3 Transduction

A mid-exponential culture of kanamycin sensitive *Rp. blastica* was subcultured and grown to a concentration of  $5 \times 10^8$  cells  $\text{ml}^{-1}$  (OD 1.6, 650 nm). Phage, prepared from kanamycin resistant cells, were added to an moi of 0.1 and incubated for 30 or 50 min.  $100 \mu\text{l}$  aliquots were then spread onto PMY plates containing  $50 \mu\text{g ml}^{-1}$  of kanamycin. As a control kanamycin sensitive *Rp. blastica* were spread on PMY to determine the level of spontaneous Km resistant mutants.

### 2.28.4 Ultra-violet inactivation of ØBHG1

5 ml of ØBHG1 ( $5 \times 10^{10}$  pfu  $\text{ml}^{-1}$ ), propagated on Km resistant *Rp. blastica* were exposed to short wave ultra violet radiation (170 ergs  $\text{cm}^{-2}$ ) for varying time intervals (Goldschmidt and Landman., 1961). Transduction was checked as described in section 2.28.3 with the moi varying depending on the sample used (moi was based on the titer prior

to irradiation) as shown below:

<u>Time of exposure</u>	<u>a viable phase</u>	<u>mol used in</u>
(sec)		<u>transduction</u>
0	100	0.1
30	5	1
45	1	1
60	$3.75 \times 10^{-1}$	5
120	$1.75 \times 10^{-3}$	10
240	$2.5 \times 10^{-8}$	10

## 2.29 Transfection

### 2.29.1 Method 1 - freeze/thawing

Transfection was attempted using the technique of Holsters *et al.*, (1978). A chemoheterotrophically grown, overnight culture of *Rp. blasticus* was diluted to an OD ( $A_{650}$ ) of 0.8, ( $1 \times 10^8$  cells  $\text{ml}^{-1}$ ). Incubation was continued until a final OD of 1.6 ( $A_{650}$ ), corresponding to  $5 \times 10^8$  cells  $\text{ml}^{-1}$ , was reached. 200 ml were removed, washed with 0.01 M Tris-HCl (pH 7.4-7.6) and concentrated to  $1 \times 10^{10}$  cells  $\text{ml}^{-1}$ . 200  $\mu\text{l}$  of the concentrated bacterial suspension were mixed with 100  $\mu\text{l}$  of  $\phi\text{BHC1}$  DNA of differing concentrations (0, 0.1, 1, 10, 30, 50 and 70  $\mu\text{g}$  DNA) made up in PMY medium and the lot frozen for 5 min at  $-70^\circ\text{C}$ . Samples were subsequently thawed at  $37^\circ\text{C}$ , incubated for a further 25 min, diluted two-fold in PMY, then plated by the agar-layer method. Plates were observed after 24 and 48 hr incubation. Dilutions were also plated on PMY to determine the total number of viable bacterial cells.



#### 2.29.2 Method II - polyethylene-glycol

Polyethylene-glycol induced transfection of *Rp. blastica* was attempted by the method of Klebe *et al.*, (1983), as described for the genetic transformation of *E. coli*, with the following changes. Cells were harvested on reaching a concentration of  $5 \times 10^8$  cells and the PEG 1000 was obtained from Koch-Light. The amounts of ØBHG1 DNA used were 0.1, 1, 5, 10 and 20 µg. Samples were plated by the agar layer method and the total number of viable bacteria was determined.

#### 2.29.3 Method III - Tris-HCl

The technique of Fornari and Kaplan (1982), whereby *Rb. sphaeroides* was made competent by washing with a Tris-HCl solution, was applied to *Rp. blastica*. 20 ml of a mid-exponential ( $5 \times 10^8$  cells ml<sup>-1</sup>) chemoheterotrophically grown *Rp. blastica* culture were pelleted and washed once in 10 ml of ice cold, 500 mM Tris (pH 7.2). The pellet was resuspended in 1.2 ml of ice-cold 100 mM Tris-HCl (pH 7.2), 200 mM CaCl<sub>2</sub>. Varying amounts of ØBHG1 DNA 0.1, 1, 5, 10 and 40 µg were added immediately to 0.2 ml of competent cells, and an equal volume of cold 40% PEG-6000 (BDH Chemicals) in 100 mM Tris-HCl (pH 7.2) was added slowly. The suspension was gently mixed, left on ice for 10 min, then heat shocked at 35°C for 2 min. This was followed immediately by the addition of 1.0 ml of PMY and further incubation at 35°C for 20 min. The contents of the tube were then plated by the top agar layer method and the number of viable cells determined.

## RESULTS and DISCUSSION

### 3. Phage Characterisation

#### 3.1 Phage Characteristics

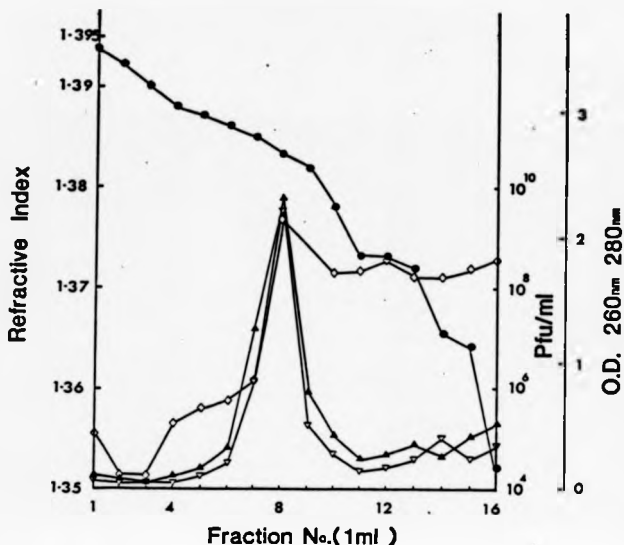
Characterisation of phage ØBHG1, isolated from a small autotrophic pond, was undertaken:

##### 3.1.1 Phage isolation

Isolation of ØBHG1 by caesium chloride density gradient centrifugation gave a single opalescent band. Optical density measurements (260 and 280 nm) and titration of gradient fractions showed this to correspond to a phage band at a density of  $1.3850 \text{ g cm}^{-3}$  (Fig. 8). Occasionally, however, two phage bands were observed. The upper band, being approximately five-fold less in concentration, was designated ØBHG2 and the lower as ØBHG1 with buoyant densities of  $1.3838 \text{ g cm}^{-3}$  and  $1.3850 \text{ g cm}^{-3}$  respectively.

To determine the nature of the difference in the buoyant densities a number of physical characteristics of ØBHG1 and ØBHG2 were studied including morphology (section 3.1.2), phage DNA analysis (section 3.1.3) and protein analysis (section 3.1.4). Both phage bands contained viable phage and electron microscopy studies, protein analysis and DNA analysis indicated that the bacteriophage were indistinguishable.

Weigle *et al.* (1959) reported density alterations of bacteriophage  $\lambda$  associated with changes in the amount of DNA packaged, e.g. a deletion of 800 base pairs gave a change of  $0.060 \text{ g cm}^{-3}$  in the buoyant density of the phage particle. Restriction fragment analysis of ØBHG1 and ØBHG2 DNAs (section 3.1.3) showed no obvious deletions under the conditions used, but does not rule out a small difference in the amount of DNA



**Figure 8**

Fractionation of a caesium chloride gradient used in the determination of the buoyant density of  $\phi$ BHCl.

Caesium chloride was added to give an average density of  $1.546 \text{ g cm}^{-3}$ . Samples were centrifuged at  $230,500 \text{ g}$  at  $10^\circ\text{C}$  using a SW40 T1 Beckman rotor. 1 ml fractions were collected from the bottom. ●----● refractive index; ◇----◇ pfu ml<sup>-1</sup>; ▲----▲ OD at 280 nm; ▽---▽ OD at 260 nm.

packaged giving rise to the two phage types. Weigle *et al.* (1959) also suggested a number of other factors that might result in altered buoyant densities of phage particles including changes in the intrinsic density, packing or salvation of the DNA, none of these possibilities being exclusive of the other.

Similar observations have been made with phage F1 when isolated by caesium chloride density gradient centrifugation (Sternberg *et al.*, 1977), the upper band being approximately 90% of the total phage population. No physical difference between the phage of the two bands could be determined and, indeed, both bands were taken to be identical and pooled.

To ensure phage homogeneity only phage of the buoyant density  $1.3850 \text{ gcm}^{-3}$  were used and redesignated as ØBHG1.

### 3.1.2 Phage morphology

ØBHG1 and ØBHG2 were found to be identical in morphology. The phage has a polyhedral head of 62 nm and a short tail 39 nm long (Fig. 9). A number of tail appendages could be observed, including a possible collar near the head of the phage and a basal plate with short spikes. No tail fibres could be seen and the tail appeared to be non-contractile.

ØBHG1 belongs to Group C in Bradley's scheme of classification for bacteriophage (Bradley, 1967). It is an average sized phage which is unlike any other phage isolated and characterised for the members of the *Rhodospirillaceae* (Table 1). The morphology of phage for the *Rhodospirillaceae* being highly variable belong to Group's A, B and C.

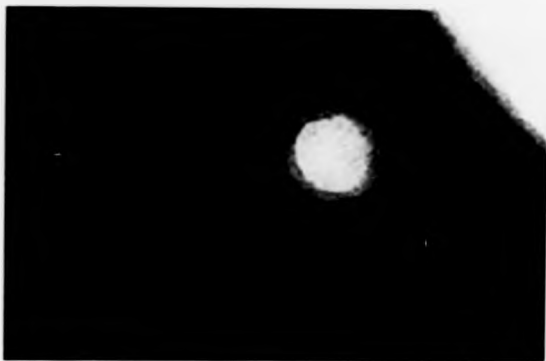


Figure 9      Electron micrograph of ØBHG1.

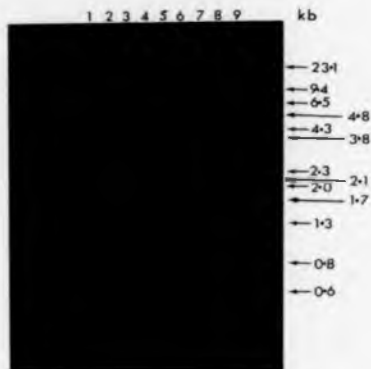
Negatively stained with 0.2% (w/v) phosphotungstic acid. The bar represents 100 nm.

### 3.1.3 DNA characterisation

When analysed by agarose gel electrophoresis DNA from ØBHG1 and ØBHG2 gave a similar discrete band (Fig. 10) with a molecular weight of 48 kb. The discrete band of the ØBHG1 genome showed no size heterogeneity as has been reported for the phage RS1 of *Rb. sphaeroides* (Donohue *et al.*, 1985).

Ethanol precipitation of ØBHG1 and ØBHG2 DNA failed to remove all impurities. A further precipitation with isopropanol was necessary to allow endonuclease digestion suggesting the possible presence of contaminating polysaccharide. EcoRI endonuclease digestion of the phage genomes gave a complex restriction pattern with fragments ranging from 5 kb to 0.4 kb, with no apparent differences between ØBHG1 and ØBHG2 to explain the different buoyant densities of the phage. This, however, does not rule out a possible minor deletion in one of the larger restriction fragments from one of the phage, and only a more complex series of restriction digests could determine any differences.

The buoyant densities of both phage DNA's were identical ( $1.7235 \text{ g cm}^{-3}$ ) corresponding to a G+C content of 50.6 mol%. The G+C content of *Rp. blastica* DNA is 65.3% (Eckersley and Dow, 1980). Differences in the G+C content between bacteriophage and their corresponding host are common among the *Rhodospirillaceae*, e.g. *Rb. sphaeroides* 2.4.1 has a G+C content of 67 mol%, while two of its phage ØRSG1 and ØRS1 have values of 46 and 71.8% respectively (Abeliovich and Kaplan 1974; Duchrow *et al.*, 1985). This is in contrast with some phage isolated for *Caulobacter*, e.g. the G+C content of the phage ØBK and its host *C. crescentus* are 65 mol% and 67 mol% respectively (Agabian-Keshishian and Shapiro, 1970).



**Figure 10** Analysis of ØBHG1 and ØBHG2 DNA.

Samples were loaded onto a 0.7% (w/v) agarose gel with TBE buffer and  $1 \mu\text{g ml}^{-1}$  of ethidium bromide. Lane 1, 1  $\mu\text{g}$  of  $\lambda$  DNA restricted with Hind III; Lane 2, 1  $\mu\text{g}$  of pC01 restricted with Pst I and EcoRI; Lane 3, 2  $\mu\text{g}$  of ØBHG2 DNA restricted with EcoRI; Lane 4, 2  $\mu\text{g}$  of ØBHG1 DNA restricted with EcoRI; Lane 5, 1  $\mu\text{g}$  of pC01 restricted with Pst I and EcoRI; Lane 6, 1  $\mu\text{g}$  of ØBHG2 DNA unrestricted; Lane 7, 1  $\mu\text{g}$  of ØBHG1 DNA unrestricted; Lane 8, 1  $\mu\text{g}$  of  $\lambda$  DNA unrestricted; Lane 9, 1  $\mu\text{g}$  of ØBHG1 DNA restricted with Hind III. pC01 was donated by Chris Oakley.



#### 3.1.4 Protein analysis

SDS-PAGE of both ØBHG1 and ØBHG2 showed ten capsid proteins (Fig. 11), with molecular weights ranging from 98,000 to 18,000. C<sub>5</sub> and C<sub>6</sub> appeared to be the major capsid proteins.

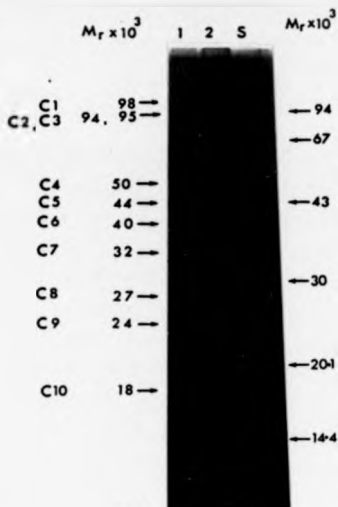
No obvious differences in the protein content of ØBHG1 and ØBHG2 were observed (Fig. 11). The sum of the molecular weights of the capsid proteins was approximately  $5.22 \times 10^5$  corresponding to approximately 26% of the coding capacity of the phage DNA.

#### 3.1.5 Lipopolysaccharide hydrolase

Many tailed phages from Bradley's Group C adsorb to lipopolysaccharide (LPS) or exopolysaccharide (EPS) receptors on the surface of the bacterium and have been found to contain LPS and EPS hydrolases. The enzyme activity has been found to be a structural protein of the tail, often a subunit of the base plate or spikes (Kanegasaki and Wright, 1973; Riegler *et al.*, 1975).

No free sugars were detected after aniline phthalate staining of LPS incubated in the presence of ØBHG1 then run on TLC plates. When ØBHG1 was incubated with LPS there appeared to be a reduction in the viscosity of the solution, but there was no apparent precipitate or sediment formed. The latter is often associated with release of lipid A from LPS and the former with the cleavage of the oligosaccharide chains.

This data must however be interpreted with care since the absence of any stainable material on these TLC plates, except for the standard sugars, suggests that the stain may have been incapable of staining oligosaccharides. Hydrolases of LPS and EPS often produce trisaccharides



**Figure 11** Page analysis of presumptive capsid protein of  
 SBHG1 and SBHG2

Samples were purified via two caesium chloride gradients and denatured by boiling for 3 min in sample buffer. Lane 1,  $10^9$  pfu of SBHG2; Lane 2,  $10^9$  pfu of SBHG1; Lane 3, molecular weight standards. Proteins were stained by silver nitrate.

and larger oligosaccharides. Unless free sugars or disaccharides were produced no breakdown products would be detected. (An alternative approach would be to use alkaline silver nitrate staining).

### 3.1.6 Conclusions

- a) Buoyant density of phage particle -  $1.3850 \text{ g cm}^{-3}$ .
- b) Morphology: 62 nm polyhedral head, short tail 39 nm long, collar, base plate and spikes, non-contractile tail. Belongs to Group C in Bradley's scheme of classification.
- c)  $\phi$ BHCl DNA: 48 kb in size, buoyant density  $1.7235 \text{ g cm}^{-3}$ , G+C content 50.6 mol%.
- d) Ten capsid proteins, molecular weights ranging from 98,000 to 18,000.

### 3.2 Optimisation of the Efficiency of Plaquing

The optimisation of the efficiency of plaquing (EOP), defined as the number of plaques under one set of conditions divided by the number of plaques under standard conditions, was essential for the titration of phage throughout this project.

#### 3.2.1 Temperature

The effect of temperature on the EOP of  $\phi$ BHCl on *Rp. blasticus* was as shown in Figure 12. Between  $15^{\circ}\text{C}$  and  $30^{\circ}\text{C}$  there was a rise of 59% in EOP which dropped rapidly by 98% when the temperature of incubation was increased to  $37^{\circ}\text{C}$ .

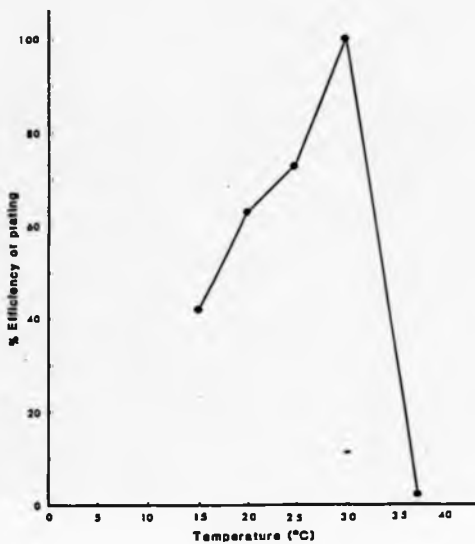


Figure 12

The effect of temperature on the efficiency of plaquing.

$10^2$  pfu of 229E1 was plated and incubated at various temperatures.

The EOP of ØBHG1 therefore has a narrow optimum temperature range, the maximum occurring at 30°C, corresponding to the optimum temperature for growth of *Rp. blastica*. This temperature, i.e. 30°C, appears to be the optimum EOP temperature for all the phage of the *Rhodospirillaceae* so far isolated. It is interesting to note that in the natural environment such temperatures would normally not be reached. Thus the optimal conditions chosen are not necessarily a true reflection of environmental bacteria/phage interactions.

Seeley and Primrose (1980) noted that some coliphage did not show co-incidence of the temperature for maximum EOP of the phage and the maximum specific growth rate of the host. They showed that coliphages fell into three physiological classes depending upon the effect of temperature on their EOP. These three classes were called low temperature (LT), mid-temperature (MT) and high temperature (HT). The maximum and minimum plating temperatures appeared to be stable properties of the virus and were not influenced by the temperature for growth of the host. Relatively little is known about the effect of temperature on phage multiplication. Temperature could affect one or more of the following stages: adsorption, eclipse, penetration and multiplication. Bacteriophage  $\lambda$ , E<sup>15</sup> and ØX174 were typical MT phages, but low temperatures affected their host cell interaction in different ways. With phages  $\lambda$  and E<sup>15</sup>, decreasing temperatures decreased the proportion of phage particles which injected their DNA, whereas with phage ØX174 low temperatures inhibited eclipse and the latter stages of replication (Primrose *et al.*, 1982). However, phage of the *Rhodospirillaceae*, including ØBHG1, appear to differ in that the temperature for optimal EOP is directly related to the temperature for maximum specific growth rate of their hosts, i.e. 30°C.

#### 3.2.2 Growth physiology

The EOP of ØBHG1 appeared to be independent of the mode of growth (chemoheterotrophic or photoheterotrophic) of the host *Rp. blastica*. Similar observations have been reported for phages of other members of the *Rhodospirillaceae* (Table 1). As it proved more difficult to reproducibly ensure complete lawns with *Rp. blastica* grown photoheterotrophically, all plaque assays were performed using chemoheterotrophically grown cells.

#### 3.2.3 Culture age

The EOP was unaffected when identical cell concentrations of mid-exponential and stationary phase cultures of chemoheterotrophically grown *Rp. blastica* were used for titration of ØBHG1. However, there did appear to be a slight decrease in plaque size with stationary phase cultures (section 4.3. ).

#### 3.2.4 Conclusions

- a) The temperature for optimal EOP of ØBHG1 was 30°C, the same temperature for the maximum specific growth rate of *Rp. blastica*.
- b) EOP of ØBHG1 appeared to be independent of the growth conditions of *Rp. blastica*, i.e. photoheterotrophic or chemoheterotrophic.
- c) The phase of growth of *Rp. blastica* did not affect the EOP of ØBHG1.

#### 3.3 Parameters Affecting the Rate of Adsorption

Factors affecting the efficiency of adsorption of ØBHG1 to *Rp. blastica* were investigated to allow standard conditions to be selected for future experiments.

### 3.3.1 Addition of cations

Figure 13 indicates that ØBHG1 appeared to have no specific cation requirement. The rate of adsorption of ØBHG1 to *Rp. blasticus* cells was identical whether or not the buffer in which the cells were resuspended was supplemented with  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $NH_4^+$ , or  $Na^+$  cations. These observations were further supported by data on the effects of increased concentrations of cations on the EOP. An increase in the concentration of  $Mg^{2+}$  or  $Ca^{2+}$  resulted in no increase in the EOP of ØBHG1.

The role of cations is clearly seen for the coliphage, e.g. T1 requires a concentration of 0.001 M divalent or 0.01 M monovalent cations for optimal adsorption. This requirement for cations probably arises from the need to neutralise electrostatic repulsive forces which would otherwise prevent the approach of negatively charged virus particles to the overall negatively charged bacterial surface (Puck and Tolmach, 1954). It is possible that cations may also bring about some subtle structural changes in the phage required for the adsorption process. Although ØBHG1 showed no cation requirement for adsorption this is not found with all the phages of the *Rhodospirillaceae*, e.g. ØRC1 has been found to have a specific requirement for  $Ca^{2+}$  and  $Mg^{2+}$  but not for the monovalent cations  $NH_4^+$  or  $Na^+$  (Schmidt *et al.*, 1974).

### 3.3.2 Organic factors

Although individual organic cofactors were not tested, the observation that the rate of adsorption of ØBHG1 was identical in Tris-HCl buffer or PMY indicated the absence of such a requirement (Fig. 13).

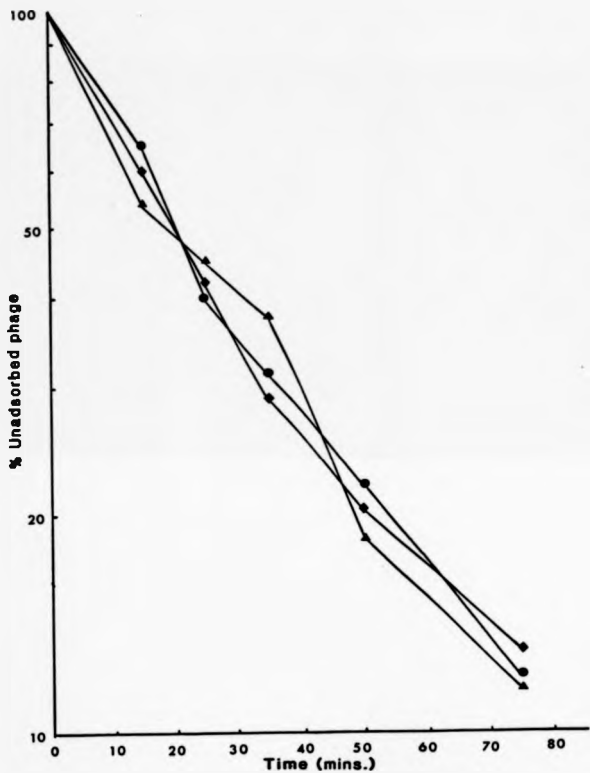
Such a requirement has been reported for the coliphage T4 and T6 where L-tryptophan acts as an adsorption factor. Several other substances

Figure 13

The effect of varying the adsorption medium on the rate of irreversible adsorption of  $10^7$  pfu  $\text{ml}^{-1}$  of  $\phi\text{BHG1}$  to  $5 \times 10^8$  cells  $\text{ml}^{-1}$  of chemoheterotrophically grown *Rp. blastica* at  $30^\circ\text{C}$ .

●---●, 0.01 M Tris-HCl, pH 6.8; ◆---◆, 0.01 M Tris HCl, pH 6.8 supplemented with NaCl,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{NH}_4\text{Cl}$ ,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  to final concentration of 10 mM, 10 mM, 10 mM and 3 mM respectively; ▲---▲ PMY medium.





including phenylalanine, di-iodotryptosine, tyrosine and norleucine are also effective at higher concentrations, but D-tryptophan is inactive (Stent, 1963). L-tryptophan appears to interact with the tail fibres causing relaxation at the tail sheath, resulting in the tail fibres being out-stretched so they can engage the receptor sites on the bacterial surface (Brenner *et al.*, 1962). None of the phages for the *Rhodospirillaceae* appear to require any organic cofactors for adsorption. This might be expected since only  $\phi$ RC1 (Schmidt *et al.*, 1974) appears to possess complex tail appendages.

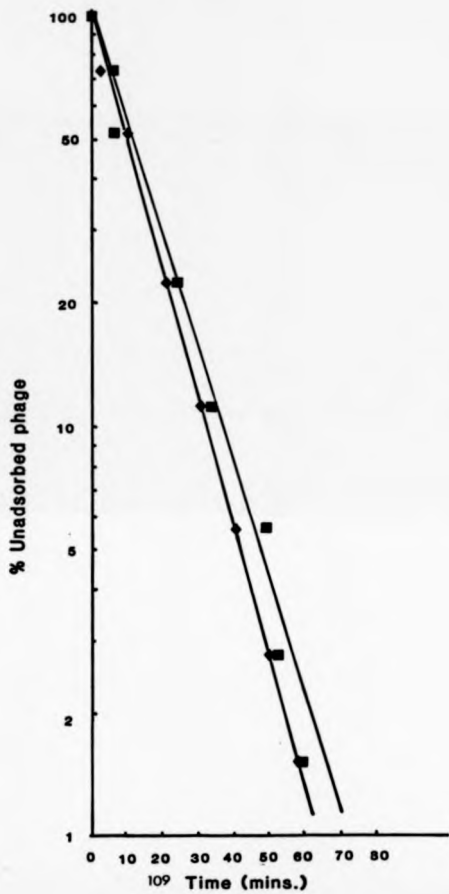
### 3.3.3 Growth physiology

Adsorption of  $\phi$ BHG1 appeared to be identical for both chemoheterotrophically and photoheterotrophically grown cells (Fig. 14), the adsorption constant  $k$  was determined as  $1.39 \times 10^{-9} \text{ ml min}^{-1}$ . The constant is a measurement of both reversible and irreversible adsorption.

Although the rate of adsorption of  $\phi$ BHG1 has been found to be independent of the mode of growth of *Rp. blastica*, RS1, a phage specific for *Rb. sphaeroides*, has shown some physiological specificity (Abeliovich and Kaplan, 1974). Photoheterotrophic cells appeared to a large extent immune to infection with RS1. This partial immunity may represent some changes in the structure or composition of the surface of the cells, these changes causing difficulties in the adsorption or penetration process. Later, Deal and Kaplan (1983b) using immunochemical studies, reported that there appeared to be no significant difference in the amounts of major outer membrane proteins with changes in the mode of growth of *Rb. sphaeroides*. However, this does not eliminate possible changes in proteins and other cell wall

Figure 14

The rate of reversible and irreversible adsorption of  
 $10^7$  pfu  $\text{ml}^{-1}$  of  $\phi\text{BHG1}$  to  $5 \times 10^8$  cells  $\text{ml}^{-1}$  of  
chemoheterotrophically  $\blacksquare$  and photoheterotrophically  
 $\blacklozenge$  grown *Ep. blastica* at  $30^\circ\text{C}$



constituents that are masked from the antibodies.

#### 3.3.4 Cell concentration

The rate of phage adsorption appeared to increase proportionately to the concentration of *Rp. blastica* when below  $5 \times 10^8$  cells  $\text{ml}^{-1}$ . However, above this concentration the rate of adsorption appears to be constant (Fig. 15).

Stent (1963) noted a similar result with T4 and *E. coli*. He also observed that the rate constant of adsorption for T4 decreased by more than a factor of 10 if the temperature of the adsorption mixture was lowered from 25°C to 5°C. These two observations led to the idea that phage adsorption involves at least two steps, and that the second step was rate-limiting at high bacterial concentrations and sensitive to temperature changes.

Several theories have been put forward to explain this phenomenon. The first is that each phage particle oscillates between one of two states (Fig. 16a). An "active state", in which every collision with a receptive bacterium leads to irreversible adsorption, and an "inactive state", in which no collision can lead to adsorption. At very high bacterial concentrations the "active" fraction of the phage population becomes irreversibly fixed almost at once and the remainder of the phages can be absorbed no faster than the rate at which they can make the transition from the "inactive" to "active" state.

The second postulate (Fig. 16b), is that the phage particles can collide with bacteria in two different ways, one 'good', which adsorbs the phage irreversibly and one 'bad' which fixes the phage reversibly to the

Figure 15      The effect of differing the concentration of *Rp.*  
*blastica* cells on the rate of ØBHG1 adsorption.

$5 \times 10^6$  pfu  $\text{ml}^{-1}$  of ØBHG1 was added to varying cell concentrations,  
after 30 min incubation the number of unadsorbed phage was determined.

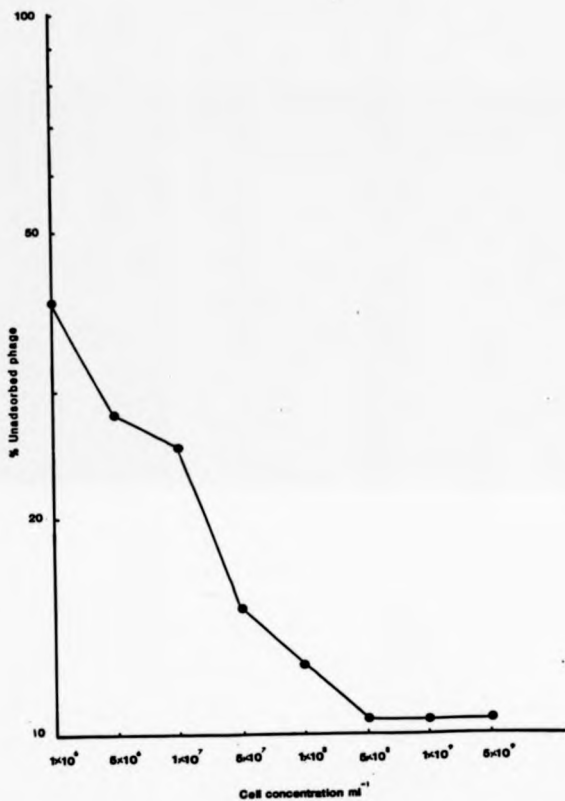


Figure 16

The three models envisioned by Stent (1963) for the nature of the second step in phage adsorption.

- a)  $k_1$  - maximum adsorption rate at high cell densities

$$k_1/(k_1+k_2) - f$$

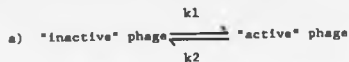
- b)  $k_3f$  - maximum adsorption rate at high cell densities

$$k_1/(k_1+k_3) - f$$

- c)  $k_3$  - maximum adsorption rate at high cell densities

$$k_3/(k_2+k_3) - f$$





+

bacterium

↓

no reaction

+

bacterium

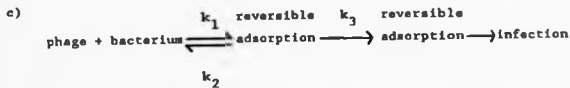
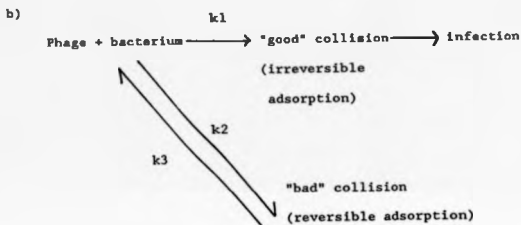
↓

irreversible

adsorption

↓

infection



surface of the cell without allowing it to become irreversibly adsorbed. At high bacterial concentrations, nearly all the phages become immediately attached to cells. However, only a minor fraction of the particles is initially absorbed in the 'good' or irreversible way, the rate of irreversible adsorption of the remainder being limited by the rate of desorption of the reversibly held particles.

Finally, it is possible that at each collision, phage and bacteria enter a potentially reversible attachment state (Fig. 16c). While held to the cell surface, the phage may either become fixed irreversibly, or it may desorb from the bacterium before becoming irreversibly fixed. At high bacterial concentrations, nearly all the phage particles immediately enter the reversible adsorbed state, so that the rate of irreversible fixation at the bacterial surface becomes the rate-limiting reaction in the overall adsorption velocity.

### 3.3.5 Conclusions

- a) Cations or organic cofactors were not required for ØBHG1 adsorption.
- b) The rate of adsorption of ØBHG1 was independent of the growth conditions of *Rp. blastica*.
- c) The maximum rate of adsorption was reached at a cell concentration of  $5 \times 10^8$  cells  $\text{ml}^{-1}$ .

#### 4. Bacterial/Phage Interactions

##### 4.1 Bacterial Receptor Sites for ØBHG1

Electron micrographs (Fig. 17a) clearly showed that ØBHG1 attached to one specific pole of *Rp. blastica*. Further studies suggested that ØBHG1 adsorbed specifically to the "old pole" and not the "new". Phage can be seen attached to both poles when, at some defined point, both poles can be designated as "old". The bacteriophage appeared not to adsorb to the actively growing pole or plane of division (Fig. 17a). The relationship between phage adsorption and the *Rp. blastica* cell cycle is given in Fig. 17a. There appeared to be some macro-molecular differentiation of the cell surface of *Rp. blastica* by which ØBHG1 attached only to the "old pole".

Only one other phage for the *Rhodospirillaceae* has been reported to have a specific site for adsorption - Rpl, specific for *Rhodopseudomonas palustris* la5. This adsorbs only to one pole (Bosecker *et al.*, 1972b). Comparisons of the adsorption of this phage to that of ØBHG1 during the hosts cell cycle (Figs. 17a and 17b) show that Rpl is restricted to the area of active growth, i.e. the new or dividing pole, and not the old pole as found with ØBHG1 and *Rp. blastica*. ØBHG1 and ØRpl are therefore 'markers' of macromolecular differentiation of the cell surfaces of their respective hosts.

##### 4.2 Host Range

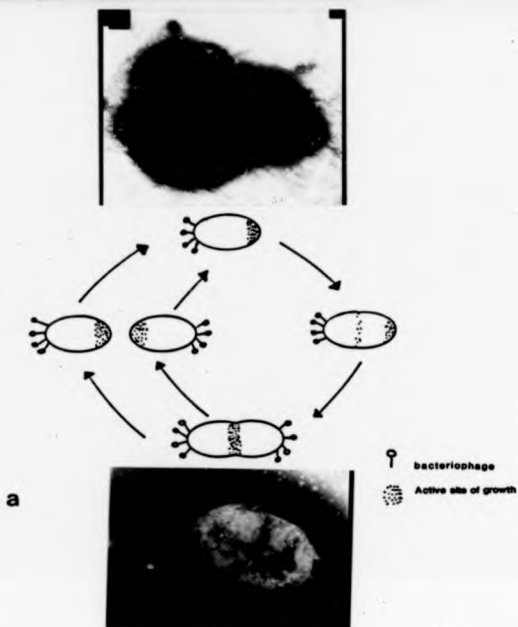
ØBHG1 was found to replicate only on *Rp. blastica* (Table 2), but non-specific lysis or 'lysis from without' occurred with *Rb. sphaeroides* 8253 on spotting  $10^8$  pfu of ØBHG1. The resulting zones of lysis when cored

Figure 17a

The relationship between the sites of phage ØBHG1 attachment on *Rp. blastica* cells and the bacterium cell cycle

Figure 17b

The relationship between phage Rpl adsorption and the cell cycle of *Rhodopseudomonas palustris*.  
Taken from Borecker et al. (1972).



and eluted showed that *Rb. sphaeroides* 8253 failed to support growth of ØBHG1 on both agar lawns and liquid cultures. No interaction was found with any other member of the *Rhodospirillaceae*.

The narrow host range of ØBHG1 is typical for the phage of the *Rhodospirillaceae*, but there are no other reports of non-specific lysis as found with ØBHG1. The non-specific lysis of *Rb. sphaeroides* 8253 by ØBHG1 suggests a relatedness to *Rp. blastica* not observed by Imhoff *et al.* (1984) in their recent reclassification of the *Rhodospirillaceae*.

#### 4.3 Plaque Morphology

Plaque morphology for ØBHG1 was highly variable with clear round plaques ranging in size from less than 0.5 mm to 1.5 mm in diameter. An occasional slight halo could be seen when stationary growth phase cultures of *Rp. blastica* were used for titration. Since halos were observed only occasionally and were small ( $\leq$  0.3 mm wide) this suggested that they were due to released cellular metabolites rather than a phage encoded lysis or lysozyme. After coring individual large and small plaques and replating the eluted phage, the plaques in all cases were of variable size. Thus, the size of the plaque was not a genetic determinant of the phage.

#### 4.4 One-Step Growth Curves

As described in the introduction (section 1.3.3) two methods have been used to prevent further phage attachment during one-step growth curves, i.e. cations and antibodies. The removal of cations essential for adsorption to prevent further adsorption of phage has been used for two

*Rhodospirillaceae* phage-RC1 (Schmidt *et al.*, 1974) and RS1 (Abeliovich and Kaplan, 1974). However, this approach could not be attempted with ØBHG1 since this phage showed no cation requirement for adsorption (section 3.3.1). Consequently, antibodies had to be used to allow an accurate one-step growth curve analysis.

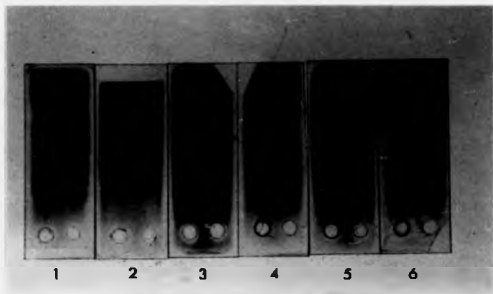
#### 4.4.1a Antibodies to ØBHG1

Figure 18 shows the immuno-rocket electrophoresis plates used to detect antibodies to ØBHG1 in crude antisera. The lack of coomassie stained peaks for pre-immune serum indicated that peaks obtained with post-immune serum were indeed due to antibodies raised specifically against ØBHG1.

#### 4.4.1b One-step growth curves with phage antisera

A concentration of antiserum was required that would inactivate  $10^7$  pfu  $\text{ml}^{-1}$  of ØBHG1 (the concentration of phage used in the one-step growth curves) within 5 min. From Table 5 it was apparent that antiserum diluted 1:50 gave complete inactivation, and further dilutions failed to inactivate all ØBHG1 present.

Figure 19 shows the one-step growth curves obtained and the duration of the various phases. The conditions of growth chosen for *Rp. blasticus*, i.e. photoheterotrophic or chemoheterotrophic had no effect on the length of the latent and rise periods and little effect on the final burst size. Table 6 gives various parameters of one-step growth curves for phage of other members of the *Rhodospirillaceae*. ØBHG1 is comparable with this data in having a relatively small burst size (in comparison T4 has an average burst size of approximately 100). The effect of the mode of growth on the burst size has been studied in only



**Figure 18** Immune rocket electrophoresis plates to detect antibodies in crude antisera against ØBHC1

20  $\mu$ l of  $10^8$  and  $10^{10}$  pfu  $\text{ml}^{-1}$  of ØBHC1 were solubilised in 1% (w/v) Triton X-100 and loaded into a well cut into 1% (w/v) agarose containing crude antisera. Plates 1, 2, 3 contained  $10^8$  pfu  $\text{ml}^{-1}$  ØBHC1 in the first well and  $10^{10}$  pfu  $\text{ml}^{-1}$  ØBHC1 in the second well with agarose containing 0.5, 1 or 5% (w/v) pre-immune serum respectively. Plates 4, 5, 6 contained  $10^8$  pfu  $\text{ml}^{-1}$  ØBHC1 in the first well and  $10^{10}$  pfu  $\text{ml}^{-1}$  ØBHC1 in the second well with agarose containing 0.5, 1 or 5% (w/v) post immune serum respectively. Plates were stained with coomassie blue.



Table 5

Effects of dilution of antisera to ØBHG1 in the  
inactivation of  $10^7$  pfu ml<sup>-1</sup> of ØBHG1 in 5 min at 30°C

Dilution of antiserum	% inactivated phage
undiluted	100
1:10	100
1:50	100
1:100	91
1:200	45

Table 6 The parameters of one-step growth curves of the Rhodospirillaceae

Host	Phage	Average burst size		Latent period (mins)		Rise period (mins)		References
		Chemo1	Photo2	Chemo1	Photo2	Chemo1	Photo2	
<u>Rb. sphaeroides</u>	R01	-	10	-	180	-	60	Mural & Friedman, 1974
	R06P	15-20	-	180	-	90	-	Pemberton & Tucker, 1977
	RS1	15-20	15-20	120	150	20	50	Abeliovich & Kaplan, 1974
<u>Rb. capsulatus</u>	RC1	51	53	120	90	60	30	Schmidt <u>et al.</u> , 1974

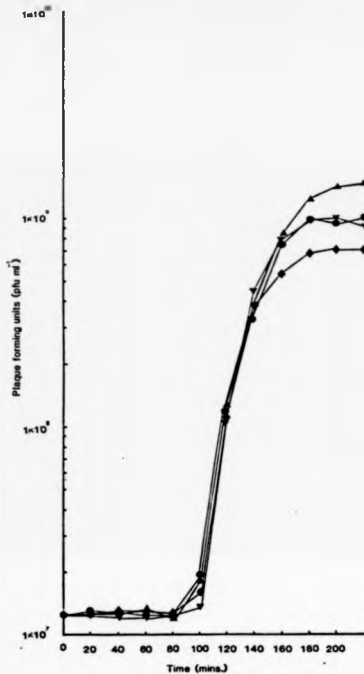
1 = Chemoheterotrophic

2 = Photoheterotrophic

Figure 12

One-step growth curves of ØBHG1 under varying growth conditions.

Exponential phase cultures of *Rp. blattica* were diluted to a concentration of  $5 \times 10^8$  cells  $\text{ml}^{-1}$ . Two protocols were followed, either phage were added, allowed to adsorb (moi of 0.02, 50 min at  $30^\circ\text{C}$ ) before the addition of phage antisera (1:50, 5 min at  $30^\circ\text{C}$ ) and dilution with pre-warmed medium followed by assaying for plaque forming units; or potassium cyanide (KCN) was added to the cells (final concentration 5 mM, 30 min at  $30^\circ\text{C}$ ) before the addition of phage (moi, 0.02, 50 min at  $30^\circ\text{C}$ ) and dilution with pre-warmed medium before assaying for plaque forming units. Under chemoheterotrophic conditions infected cells were maintained in the dark aerobically, under photoheterotrophic conditions the infected cells were illuminated  $40 \text{ Em}^{-2} \text{ s}^{-1}$  anaerobically. ◆---◆ antisera chemoheterotrophic, ●---● antisera photoheterotrophic, ▼---▼ KCN chemoheterotrophic, ▲---▲ KCN photoheterotrophic.



Growth conditions	KCN	Latent period (min)	Rise period (min)	Burst size ± standard error
chemoheterotrophic	-	80-100	100	25 ± 2.5
photoheterotrophic	-	80-100	100	30 ± 2.1
chemoheterotrophic	5 mM	80-100	100	29 ± 2.5
photoheterotrophic	5 mM	80-100	100	59 ± 15.0

two other *Rhodospirillaceae* phage RC1 and RS1 (Schmidt *et al.*, 1974; Abellovich and Kaplan, 1974), and as with ØBHG1, the burst size does not appear to be altered. However, there were differences in the latent and rise periods not observed with ØBHG1. In fact, detailed studies into the bioenergetic aspects of ØRC1 replication were made (Schmidt *et al.*, 1974) and suggested that *Rhodospirillaceae* bacterial phage systems may offer unique advantages for the study of the bioenergetics of phage growth because of the ease by which their energy systems can be manipulated with changes of growth modes. Results indicated that the energy requirement for phage replication was more stringent than that for growth of the uninfected host cell. Furthermore, continuous ATP regeneration at a high rate throughout the latent period seemed to be essential for normal virus replication.

Direct comparisons of latent and rise periods is difficult due to differing methodologies used for other phage of the *Rhodospirillaceae* but ØBHG1 appeared to have a shorter latent period and longer rise period. This may be due to the length of time needed for adsorption. Prolonged adsorption time causes initiation of infection in the population to be spread over a longer period and hence the rise period is extended.

#### 4.4.2 One-step growth curves with cell synchronisation

Attempts to synchronise the infection of *Rp. blastica* by ØBHG1 by temporarily poisoning the cells with potassium cyanide (Denhardt and Sinsheimer, 1965) had no effect on the length of time for the latent and rise periods (Fig. 19). However, although the burst size for chemoheterotrophically grown cells showed little change to that established under identical growth conditions with phage antisera

(Fig. 19), the burst size for photoheterotrophically grown cells was approximately double. The standard error for this result was  $\pm 15$  and indicated the wide range of values obtained. The increase in the value of the burst size was probably a cyanide induced phenomenon. Zamoni (1984) described a partial characterisation of chromatophores and cytochromes from *Rp. blastica*. *Rp. blastica* was found to contain a branched electron transport chain, one branch leading to a cyanide sensitive cytochrome c-oxidase and the other to a cyanide resistant form. From this it can be seen that synchronisation with cyanide was unlikely to succeed in *Rp. blastica*. The increase in the burst size under photoheterotrophic conditions suggested that for unknown reasons the addition of potassium cyanide may have resulted in a greater amount of energy available for phage replication. This effect was specific for the photoheterotrophic mode of growth.

#### 4.5 Characterisation of Spontaneous Resistant *Rp. blastica* cells

##### 4.5.1 Enumeration

The rate of spontaneous resistance to ØBHG1 expressed by *Rp. blastica* was identical whether determined in liquid culture or on the surface of agar plates. The proportion of cells hit during infection with ØBHG1 was calculated from the Poisson distribution formula (Stent, 1963), and the rate of spontaneous resistance expressed by *Rp. blastica* was calculated to be  $5 \times 10^{-6}$ . If the resistant phenotype was assumed to be due to a simple single mutational event then this would have been equivalent to a mutational frequency of  $5 \times 10^{-6}$ .

#### 4.5.2 Adsorption of ØBHG1 to resistant *Rp. blastica*

##### 4.5.2a Method I - Fluorometric

Fluorescent labelling was used in an attempt to rapidly determine whether ØBHG1 resistant *Rp. blastica* cells were capable or not of binding phage. Hantke and Braun (1974) noted that infection by phage T2-T7 caused an immediate increase in the fluorescence of cell bound ANS. The hypothesis was that phage adsorption caused a structural transition in the membrane such that ANS bound to more hydrophobic regions of proteins or dissolved more into a lipophilic phase, increased hydrophobicity leading to increased fluorescence. However, when ØBHG1 was added to *Rp. blastica* there was no significant increase in fluorescence (1%) even when ØBHG1 was added at an moi of 10. The cells were clearly labelled as a background fluorescence could be seen on excitation and this suggested that ØBHG1 adsorption did not produce fluorescence. This is unusual as enhanced fluorescence is independent of the nature of the receptor, i.e. protein or lipopolysaccharide (LPS) (Hantke and Braun, 1974) and has been successfully used on other photosynthetic bacteria (Samimi and Drews, 1978). It could be that adsorption of ØBHG1 was inhibited by the presence of the fluorescent dye or that any increased fluorescence was masked by the background fluorescence from the cells.

##### 4.5.2b Method II - Non fluorometric

Six resistant colonies of *Rp. blastica* were grown chemoheterotrophically then tested for their ability to adsorb ØBHG1 on their surfaces by determining the number of free phage remaining in suspension. Of these six, ØBHG1 failed to adsorb to four strains, but did attach to two resistant strains. This suggested that there was heterogeneity among the resistant phenotypes.

In cells where phage failed to attach, this would suggest a modification of the cell wall and a loss of activity of a phage receptor. When ØBHG1 could attach to the cell wall, resistance may have been due to failure to inject phage DNA because of cell wall modifications or intracellular modification of the host preventing ØBHG1 replication. Most commonly, such mutant bacteria are altered either in their RNA polymerase, which fails to transcribe some essential phage genes correctly, or in bacterial enzymes that participate in the processing of phage proteins during virion assembly.

#### 4.5.3 ØBHG1 lysogeny

Resistance to ØBHG1 may also be due to lysogeny of ØBHG1 where the host cell is immune to superinfection by phage particles of the same type. Loss of phage attachment has been reported after lysogeny of E<sup>15</sup>, E<sup>34</sup>, <sup>341</sup> and P22 bacteriophages for *Salmonella* due to lysogenic conversion of the O-side chains which are the receptors for these bacteriophages (Uetake et al., 1958) (section 1.3.4.1). In other systems bacteriophage can attach to the cell and infect their DNA, but the phage genome fails to replicate (Stent, 1963).

Attempts at inducing prophage from resistant cells with mitomycin C and UV irradiation were unsuccessful. UV irradiation of colonies did produce zones of clearing on the lawns below, but this was also found with sensitive *Rp. blastica* used as a control. These zones of clearing were cored but attempts to passage any phage or observe phage particles under the electron microscope were unsuccessful. These zones of growth inhibition were most likely due to the release of growth inhibitory substances from UV damaged cells.



Some prophage are not induced by mitomycin C or UV irradiation, for example, phage Mu (Howe and Bade, 1975) and phage P2 (Bertani and Bertani, 1971). The absence of a  $\phi$ BHG1 lysogen was confirmed by DNA-DNA hybridisation using labelled  $\phi$ BHG1 DNA as a probe. The autoradiograph obtained (Fig. 20) showed no detectable DNA sequence corresponding to  $\phi$ BHG1 integrated into resistant *Rp. blastica* chromosomal DNA.

#### 4.6 Lysogens Endogenous to *Rp. blastica*

Attempts to induce lysogens endogenous to wild type *Rp. blastica* were unsuccessful after exposure to mitomycin C (1, 5, 10  $\mu\text{g ml}^{-1}$ ) and UV radiation. Growth of *Rp. blastica* appeared to be reduced in the presence of mitomycin C and after ultraviolet irradiation but no lysis of cultures was apparent. Any induced lysogens of *Rp. blastica* may have failed to be titered on *Rp. blastica* due to relysogeny, and since only one strain of *Rp. blastica* has been isolated, supernatants were tested on other members of the *Rhodospirillaceae* in an attempt to find a sensitive host. Failure to detect any lysis on other members of the *Rhodospirillaceae*, and the absence of phage particles under the electron microscope indicated no easily detectable lysogen endogenous to *Rp. blastica*. Lysogens endogenous to strains of *Rb. sphaeroides* R26P, R29, R2-1 and  $\phi$ RsG1 have been identified using mitomycin C as an inducer (Tucker and Pemberton, 1980; Tucker and Pemberton, 1978; Mural and Friedman, 1974; Duchrow *et al.*, 1985).

#### 4.7 Transfection

Attempts to transfect *Rp. blastica* with  $\phi$ BHG1 DNA by freeze thawing, polyethyleneglycol and Tris-HCl were unsuccessful. These diverse

Figure 20

Detection of  $\phi$ BHG1 lysogen in spontaneous phage  
resistant *Rp. blastica* cells

a) DNA was loaded onto a 0.7% (w/v) agarose gel with TBE buffer and 1  $\mu$ g ml<sup>-1</sup> ethidium bromide. Lanes 1, 2, 4, and 5, 2  $\mu$ g of *Pst* 1 restricted DNA from spontaneous phage resistant *Rp. blastica* colonies; lane 3, 2  $\mu$ g of  $\phi$ BHG1 DNA; lane 6, 2  $\mu$ g of *Eco*RI restricted  $\phi$ BHG1 DNA; lane 7, 2  $\mu$ g of *Pst* 1 restricted *E. coli* DNA.

b) DNA-DNA hybridisation of the above gel when Southern blotted and challenged with labelled  $\phi$ BHG1 DNA. Lane 3, unrestricted  $\phi$ BHG1 DNA; lane 6, *Eco*RI restricted  $\phi$ BHG1 DNA.

1 2 3 4 5 6 7



a

1 2 3 4 5 6 7



b

methods all increase, in some way, the permeability of the cell envelope to large DNA molecules. Freeze thawing has been suggested to allow DNA molecules to penetrate, by passive diffusion, through temporary lesions in the cell wall and membrane (Dityatkin *et al.*, 1972). The modes of action of PEG treatment and the formation of competent cells with Tris-HCl are unknown.

The absence of identifiable plaques was not conclusive proof of no transfection as, for example, cell death could have been prevalent. However, this was not the case since viability remained at approximately 90%. In addition, even if  $\phi$ BHG1 DNA was taken up by the cells then phage replication may have been blocked. Had time permitted, radio-labelled  $\phi$ BHG1 DNA could be used in such transfection experiments to detect cellular DNA uptake.

The above results were not unexpected since only one example of transfection has been reported for the *Rhodospirillaceae*. Tucker and Pemberton (1980) showed that DNA extracted from bacteriophage R26P could be re-introduced into *Rb. sphaeroides* with the aid of temperate bacteriophage R29. Unfortunately, such 'helper phage' were not available for *Rp. blastica*.

#### 4.8 Transduction

Attempts to transduce a kanamycin (Km) sensitive strain of *Rp. blastica* to kanamycin resistance using  $\phi$ BHG1, grown on the kanamycin resistant strain, were unsuccessful. This may have been due to killing of the cells infected initially by free phage remaining in solution, i.e. transduction would be masked by superinfection. Initial attempts to

overcome this involved using low moles of 1, 0.1 and 0.01. Multiple infection can be prevented in some cases by the removal of cations, essential for the adsorption of the bacteriophage to cells, with the addition of chelates such as sodium citrate and EDTA. As discussed in section 3.3.1 ØBHG1 did not require cations for adsorption so chelating agents were unsuccessful. In a further attempt to prevent killing of cells, ØBHG1 was exposed to UV irradiation. Goldschmidt and Landman (1961) reported that high doses of UV reduced the plaque forming ability of the temperate bacteriophage PLT-22 but did not reduce the transduction frequency. UV irradiation also prevented normally lytic phage from entering the lytic cycle. Consequently, this procedure was useful for recovering phage-sensitive non-lysogenic transductants. Figure 21 shows the UV irradiation inactivation curve for ØBHG1. As inactivation was increased the moi was also correspondingly increased in an attempt to observe transduction, but none was detected.

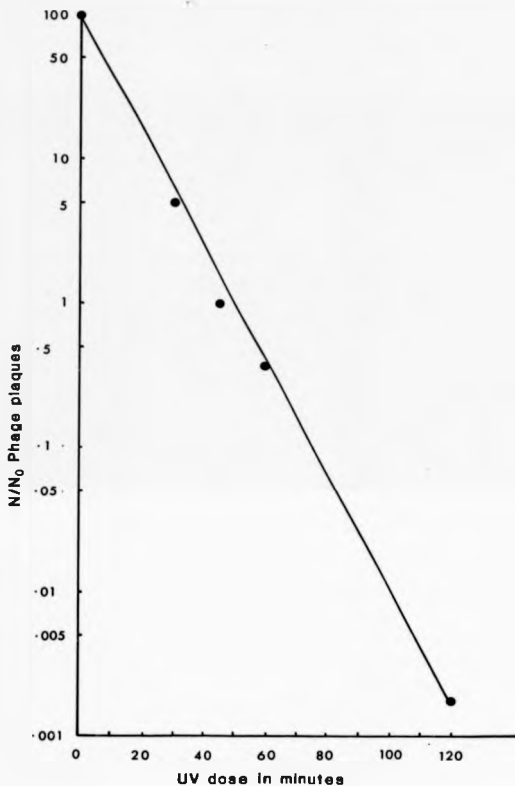
An explanation for this data can be found in that the antibiotic kanamycin interacts with ribosomes to prevent translation of mRNA. Transductants would require time to express the Km resistance gene before being exposed to the antibiotic. Consequently, 30 min was allowed for expression in these experiments, but this time may not have been sufficient.

To overcome the problems associated with selecting for an appropriate antibiotic marker, an alternative approach could have been taken. A technique to introduce transposons into *Rm. vannielii* by conjugation has recently been developed (R. Breadon, personal communication). If a transposon could be introduced into the *Rp. blastica* genome, then phage could be propagated on this strain, then screened for any that had

Figure 21

Ultra violet inactivation of ØBHG1

$5 \times 10^{10}$  pfu ml<sup>-1</sup> of ØBHG1 propagated on Km resistant *Rp. blastic* were exposed to  $170 \text{ ergs s}^{-1} \text{ cm}^{-2}$  of short wave UV radiation for varying time intervals. Samples were titred to determine the number of active plaque forming phage.



packaged the Tn using labelled Tn as a probe.

As described in the introduction, a variety of phage, both temperate and virulent have been isolated for the *Rhodospirillaceae* but none have been found to be capable of transduction. Thus, the results above were not unexpected. A form of generalised transduction does occur in *Rb. capsulatus* which is mediated by a non-infective, small phage-like particle described as a gene transfer agent (GTA).

#### 4.9 Regulation of $\phi$ BHCl Replication

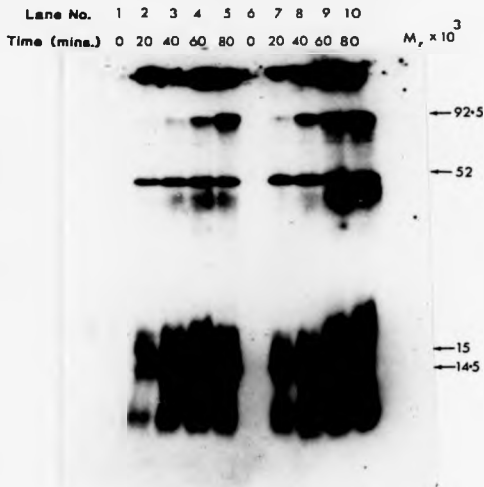
The complex control systems explicit in many bacteriophage are of great interest, primarily with respect to the temporal regulation of gene expression. Two key examples are the enzymes protein kinase and RNA polymerase.

##### 4.9.1 Protein Kinase

Protein kinases are enzymes that phosphorylate susceptible proteins and several studies have been undertaken on the protein kinases encoded by a number of bacteriophages, e.g. T7 and T3 (Pai *et al.*, 1975) and  $\phi$ Cd1 (Hedgson *et al.*, 1985). Complex control systems enable phage to develop in a highly efficient and rapid manner and protein kinases are believed to abolish host gene expression and also to terminate early gene expression during phage development. Further complexity is introduced in that with T7 (Pai *et al.*, 1975) there is an indication that a protein kinase can phosphorylate and inactivate itself.

Figure 22 shows the autoradiograph obtained after cultures of *Rp. blautica* were labelled with  $^{32}\text{P}$  ATP. The first culture was infected





**Figure 22** Attempted detection of a  $\phi$ BHG1 encoded protein kinase.

25 ml of  $5 \times 10^8$  cells  $\text{ml}^{-1}$  of *Rp. blastica* were labelled with 5 mCi of  $^{32}\text{P}$  orthoadenosine triphosphate.  $\phi$ BHG1 was added to one culture at a moi of 10, and incubated at  $30^\circ\text{C}$ . Samples were removed, washed and run on a denaturing 10% (w/v) polyacrylamide gel. Samples were solubilised in sample buffer at  $100^\circ\text{C}$  for 5 min. Lanes 1, 2, 3, 4 and 5 are uninfected *Rp. blastica* sampled at times shown. Lanes 6, 7, 8, 9 and 10 are *Rp. blastica* infected with  $\phi$ BHG1 sampled at times shown.

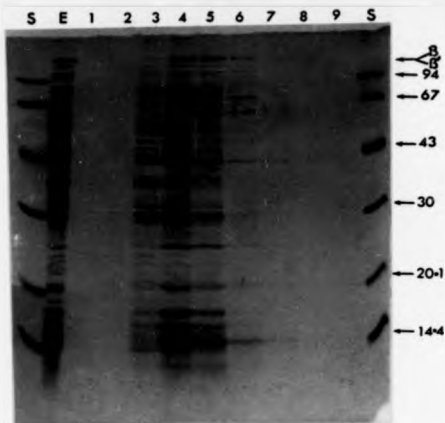
with ØBHG1 at a high moi and after 80 min four proteins with molecular weights of 92,500, 52,000, 15,000 and 14,500 were heavily labelled. However, an identical result was obtained with an uninfected control culture. These results indicate that ØBHG1 does not encode such an enzyme.

#### 4.9.2 RNA polymerase

The DNA-dependent RNA polymerase plays a central role in transcription of genetic information from DNA to RNA. Several bacteriophage encode new subunits or a complete new RNA polymerase so that only phage DNA is transcribed. Bacteriophages T3 and T4 DNAs encode an RNA polymerase consisting of a single polypeptide chain Mr 110,000 (Losick and Chamberlin, 1976), while phage SP01 DNA encodes a series of replacement sigma factors for the host RNA polymerase (Doi and Lin-Fa, 1986).

Figure 23 shows an extract of *Sp. blasticus* applied to a heparin column, fractionated by a linear salt gradient then analysed by PAGE. Fractions containing significant quantities of RNA polymerase, as demonstrated by the characteristic  $\beta\beta'$  doublet of molecular weights of approximately 98,000 and 97,500 respectively, were pooled then passed down a second column of double stranded (ds) DNA cellulose. Fractions from the linear salt gradient and strip wash of the second column when analysed by PAGE and silver stained failed to reveal any proteins.

Heparin is a highly sulphated dextrorotary negatively charged micropoly saccharide mimicking the phosphate backbone of DNA, and therefore proteins capable of binding to heparin or to DNA will be bound until removed by an increasing salt gradient. Ideally, passage down the second more specific ds-DNA cellulose column should have removed



**Figure 22** RNA polymerase enriched fraction from a linear salt gradient wash of a heparin column loaded with the soluble extract from *Rp. blasica*

50  $\mu$ l samples from 1 ml fractions of a linear salt gradient (50 mM  $\rightarrow$  1.2 M NaCl) were solubilised in sample buffer at 100°C for 3 min then run on a denaturing 10% (w/v) acrylamide gel. Staining was with coomassie blue. Lane S, standard molecular weight proteins; Lane E, whole cell extract of *Rp. blasica*

contaminating proteins and retained only DNA binding proteins, i.e. purification of the RNA polymerase. However, as described above no proteins bound to the ds-DNA cellulose column. To confirm that this column was still active, an *E. coli* cell free extract was applied directly to it. After fractionation, the results indicated that binding of proteins did occur.

It has been reported that the RNA polymerase from bacteriophage T3 is inactivated by heparin (Bautz, 1976) and similarly with RNA polymerase from *Rm. vannielii* (N. Scott, personal communication). Since proteins from *E. coli*, including RNA polymerase were capable of binding to the ds-DNA cellulose, this suggested that *Rp. blasticus* proteins were somehow inactivated by passage down the heparin column and this prevented subsequent binding.

In an attempt to overcome this problem, cell extracts were passed directly down the ds-DNA cellulose column. Figures 24 and 25 show gels of fractions resulting from the linear salt gradients of ØBHG1 uninfected and infected cell extracts respectively. From these gels it was apparent that passage down a single ds-DNA cellulose column was insufficient to purify or greatly enrich DNA polymerase as the fractions contained many other putative DNA binding proteins.

Although it was not possible to identify all of the individual, and therefore any novel, subunits associated with the RNA polymerase from phage infection of *Rp. blasticus* by this simple procedure, a number of differences in the DNA binding proteins were apparent. Infected cells of *Rp. blasticus* contained different proteins but it could not be deduced whether these were produced within the cells in response to phage

Figure 24

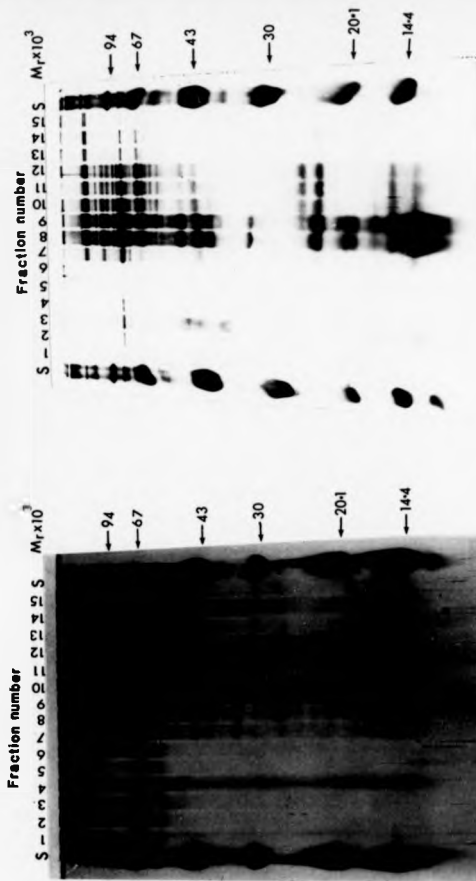
Fractions from a linear salt gradient wash of a ds-DNA cellulose column loaded with soluble extract from *Rp. blastica* cells infected with ØBHG1 at a moi of 10.

1 ml fractions from a linear salt gradient (0.1 M → 1.4 M NaCl) were dialysed against 0.01 M Tris-HCl, pH 6.8, then freeze dried. Fractions were resuspended in 50 µl of water, solubilised in sample buffer at 100°C for 5 min then loaded onto a denaturing 10% (w/v) acrylamide gel and stained with silver nitrate. Lane S, molecular weight standard proteins.

Figure 25

Fractions from a linear salt gradient wash of a ds-DNA cellulose column loaded with the soluble extract from *Rp. blastica*

1 ml fractions from a linear salt gradient (0.1 M → 1.4 M NaCl) were dialysed against 0.01 M Tris-HCl, pH 6.8, then freeze dried. Fractions were resuspended in 50 µl of water, solubilised in sample buffer at 100°C for 5 min then loaded onto a denaturing 10% (w/v) acrylamide gel and stained with silver nitrate. Lane S = molecular weight standard proteins.



infection or were phage encoded.

#### 4.10 Conclusions

- a) ØBHG1 adsorbed specifically to the 'old' pole of *Rp. blastica*.
- b) ØBHG1 replicated only in *Rp. blastica* but caused lysis from without of *Rb. sphaeroides* 8253.
- c) Plaque size was variable, 0.5 to 1.5 mm in diameter.
- d) Spontaneous resistance of *Rp. blastica* to ØBHG1 was  $5 \times 10^{-6}$ . ØBHG1 was capable of adsorption to some resistant cells. Resistance did not appear to be due to lysogeny of ØBHG1.
- e) No lysogens endogenous to *Rp. blastica* were detected.
- f) The latent period and rise period were 80 and 100 min respectively, and independent of growth physiology. The burst size was  $25 \pm 2.5$  and  $30 \pm 2.1$  for chemoheterotrophically and photoheterotrophically grown cells respectively. Synchronisation with potassium cyanide was found to affect the burst size of ØBHG1 in photoheterotrophically grown cells.
- g) Transduction and transfection were not detected under the 'conditions' given.
- h) Protein kinase was not encoded by ØBHG1.
- i) Differences between proteins that bound to a ds-DNA cellulose column from uninfected and infected cultures of *Rp. blastica* with ØBHG1 were observed, indicating the possibility of changes in the cellular RNA polymerase or the appearance of a complete new phage encoded RNA polymerase.

5. Isolation and Identification of Cell Envelope Fractions from *Rp. blasticus*

The results obtained in Section 4.1 indicated that ØBHG1 was capable of adsorbing only to the 'old pole' of *Rhodopseudomonas blasticus*. From these observations it was deduced that there must be some form of macromolecular differentiation over the bacterial surface which includes a component (or components) of the phage receptor complex. Consequently, studies were undertaken to determine the nature of the bacterial receptor for ØBHG1. These included isolation of purified cell envelope fractions from both phage sensitive and resistant *Rp. blasticus* strains and the subsequent chemical analysis of such fractions. The data obtained would provide useful information on the composition of the little-studied cell envelope of *Rp. blasticus*, and may indicate the targetting of specific cell wall components during cellular differentiation.



### 5.1 Isolation of the Outer Membrane of *Rp. blastica* (gentle lysis)

To isolate outer membranes of *Rp. blastica*, a gentle lysis technique devised for the isolation of the outer membrane of *Rs. rubrum* was used. Figure 26 shows the profile of refractive index and optical density (260 and 280 nm) obtained after sucrose gradient fractionation. There was poor separation of cellular material, and optical density readings suggested that the bulk of cellular material had failed to enter the gradient. In addition there were a number of smaller peaks which appeared randomly in different sucrose gradients (Fig. 26). SDS-PAGE gels were run in order to determine if these were membrane fragments, but as indicated in Figure 27, there was no protein present. These random peaks may have been due to other cellular material, not detectable by SDS-PAGE, such as polysaccharides or small amounts of nucleic acids not digested by deoxyribonuclease treatment.

### 5.2 Separation of Cell Envelope Fractions after French Pressing

Since gentle lysis failed to isolate the outer membrane of *Rp. blastica* an alternative approach was taken. Chemoheterotrophically grown *Rp. blastica* cells were broken by French pressing and cell debris removed by slow centrifugation. The cell envelope of *Rp. blastica* was subsequently pelleted by ultracentrifugation.

Differential sucrose density centrifugation of the cell envelope of *Rp. blastica* produced three distinct bands (Fig. 28). The top band (T), located at the 19-35% (w/v) sucrose interface was heavily pigmented and

Figure 27

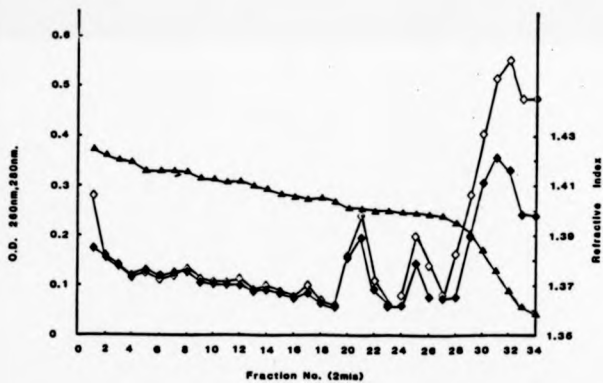
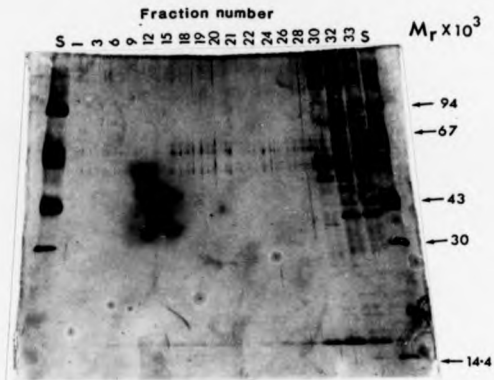
Polypeptide patterns of fractions from a linear sucrose gradient loaded with *Rp. blastica* cell envelope prepared by gentle lysis

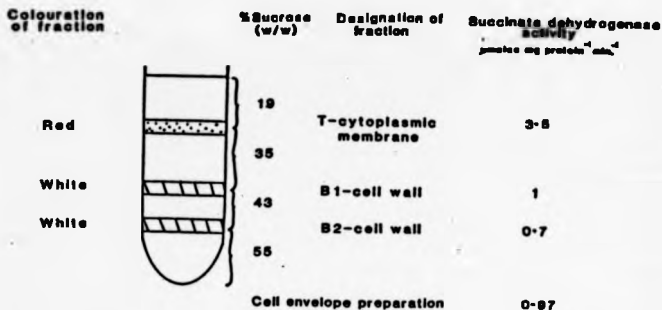
Gradients were centrifuged at 95,000 g for 13.5 hr in a Beckman SW28 rotor at 10°C. 1 ml fractions were removed by downward displacement. 50 µl of each fraction was solubilised for 5 min at 100°C in sample buffer. SDS-PAGE: 10-30% (w/v) polyacrylamide gel. Gels were visualised by silver staining.

Figure 26

Profile of refractive index and optical density (260 nm and 280 nm) obtained after differential centrifugation on linear sucrose gradients of *Rp. blastica* cell envelopes prepared by gentle lysis

Gradients were centrifuged at 95,000 g for 13.5 hr in a Beckman SW28 rotor at 10°C. ▲----▲ refractive index; ◇----◇ OD 260 nm; ◆----◆ OD 280 nm.





**Figure 28** Separation of the cell envelope of *Rp. blasticus* into cytoplasmic and cell wall fractions

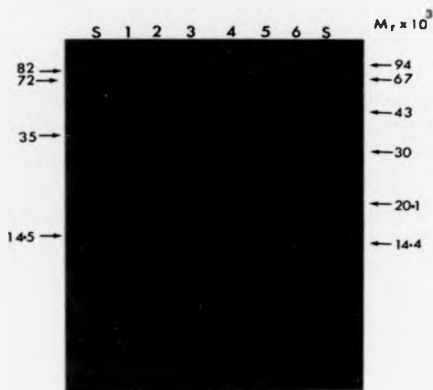
Samples were loaded onto discontinuous sucrose gradients and centrifuged at 113,000 g for 1.5 hr. The resulting fractions T, B1 and B2 were removed and assayed for succinate dehydrogenase activity, a marker of cytoplasmic membranes to determine the nature of each fraction before being applied to a second sucrose gradient.

the lower two bands, designated B1 and B2, located at the 35-43% (w/v) and 43-55% (w/v) sucrose interfaces respectively were white in colour. Improved separation of B1 and B2 was achieved by increasing the volume of 43% (w/v) sucrose.

Similar banding patterns have been reported for *Rh. capsulatus* (Flammann and Weckesser, 1984a; Bentner, personal communication), however, the lower two bands B1 and B2 in these instances were pink in colour. This was also observed with *Rp. blastica* if a culture was grown to late stationary phase, the pigment content of the cell increased and on centrifugation B1 and B2 were heavily pigmented (even after passage down a second sucrose gradient).

Separated cell-envelope fractions were further purified on second sucrose gradients. SDS-PAGE comparison of the three fractions from the first and second sucrose centrifugations showed little detectable difference (Fig. 29). Only in the B1 cell wall fraction was there an increase in the relative intensity of the predominant Mr 14,500 protein and a decrease in the intensity of the Mr 72,000 and 82,000 proteins.

Repeat preparations from different batches of cells showed slight differences in the relative intensities of the major Mr 35,000 and 14,500 protein bands after the second sucrose gradients, but there was no difference in the overall banding patterns. This was to be expected because of the variability between batch cultures and repeat preparations, but was not significant for subsequent analysis.



**Figure 29** Polypeptide patterns of cell envelope fractions  
from first and second differential sucrose density  
centrifugations

SDS-PAGE: 10% (w/v) linear acrylamide gel. Lanes 1 and 2, 30  $\mu$ g of the T band (cytoplasmic membrane) from 1st and 2nd sucrose gradients respectively; lanes 3 and 4, 30  $\mu$ g of B1 band (cell wall) from 1st and 2nd sucrose gradients respectively; lanes 5 and 6, 30  $\mu$ g of B2 band (cell wall) from 1st and 2nd sucrose gradients. Lane S, molecular weight standard proteins. Samples were solubilised in sample buffer at 100°C for 5 min. Gels were stained with coomassie blue.

### 5.3 Identification of Fractions

#### 5.3.1 Amino acid analysis

Amino acid analysis of the three bands obtained from sucrose gradients are shown in Fig. 30. The T band contained trace amounts of muramic acid (Mur) and no detectable diaminopimelic acid (DAP), suggesting the virtual absence of peptidoglycan and that this fraction was highly membrane enriched. Both B1 and B2 contained considerable quantities of Mur and DAP in the average ratios of 0.54 and 0.7 respectively (Table 7). This indicated the presence of peptidoglycan and that these bands were of cell wall origin. Ideally the ratio of Mur:DAP should be one (section 1.2.2). A lowering of the ratios could have been due to two reasons. Firstly, a decrease in the number of the tetra-amino acid chains and thus a lowering of the cross linkage between NAM units within the peptidoglycan or secondly, DAP being relatively unstable, was broken down to glutamic acid. The marked differences in the ratios of Mur:DAP and their amounts in the fractions, was most probably a true difference in the degree of cross linkage within them. This was supported by the consistent values obtained and the similar ratio of Mur:DAP found after amino acid analysis of cell wall fractions B1 and B2 from *Rp. blasticus*<sup>29</sup>.

#### 5.3.2 Succinate dehydrogenase and NADH and NADPH oxidase activity

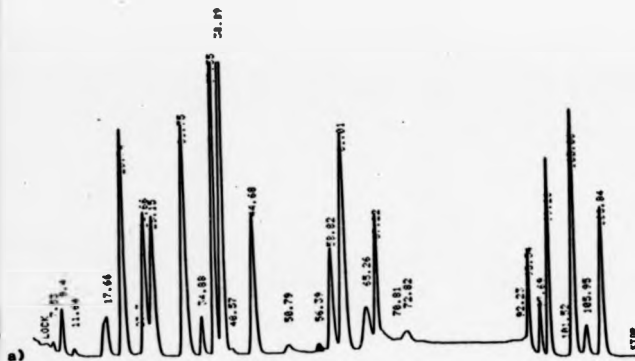
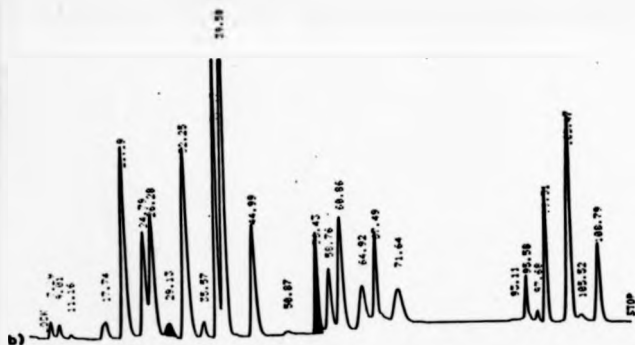
No NAD(P)H oxidase activity was present in the T, B1 or B2 fractions from the first sucrose gradients. However, when the cell envelope material was assayed before being applied to the sucrose gradient, a value of  $1.2 \mu\text{moles} \cdot \text{mg protein}^{-1} \cdot \text{ml}^{-1}$  was obtained. Thus enzyme activity was lost during centrifugation, as fractions were inactive before dialysis to remove sucrose prior to assaying. This suggested that a component of the NAD(P)H oxidase complex was loosely bound to the

Figure 30

Comparison of the amino acid analysis traces of the cell envelope fractions isolated by differential density sucrose centrifugation from *Ep. blastica*

Shaded peaks correspond to Muramic acid (Mur) at approx. 28 min and diaminopimelic acid (DAP) at approx. 56 min. a) cytoplasmic membrane; b) cell wall fraction B1; c) cell wall fraction B2.







Component	<u>Ep. blastica</u> ( $\mu\text{g mg}^{-1}$ dry wt.)			<u>Ep. blasticat<sup>9</sup></u> ( $\mu\text{g mg}^{-1}$ dry wt.)				
	cytoplasmic membrane	cell wall	B2	Triton extracted	cytoplasmic membrane	cell wall	B2	Triton extracted
<u>Neutral sugars</u>								
2-O-methyl-6-deoxyhexose	ND	ND	ND	ND	ND	ND	ND	ND
Rhamnose	1.431	2.126	2.034	ND	ND	1.902	1.5	ND
ribose	0.239	0.423	0.101	ND	ND	0.216	0.215	ND
Mannose	1.163	1.747	2.525	ND	ND	1.071	3.054	ND
Galactose	0.446	0.169	0.315	ND	ND	0.045	0.060	ND
Glucose	4.486	7.196	6.103	ND	ND	6.337	7.140	ND
<u>Fatty Acids</u>								
Cl4=0-3-oxo	3.1	3.3	3.1	0.3	3.7	1.5	2.5	0.4
Cl6=0	9.1	1.5	2.2	1.1	8.1	3.0	5.0	2.3
Cl8=0	19.3	3.4	4.1	5.7	19.8	4.9	6.3	2.3
Cl8=1	81.7	36.5	34.1	15.3	83.2	29.5	34.5	23.4
Muramic acid	0	3.344	15.61	ND	ND	ND	ND	ND
Diaminopimelic	1.933	6.19	22.3	ND	ND	ND	ND	ND

Table 7 Chemical composition of various cell envelope fractions of Ep. blastica and Ep. blasticat<sup>9</sup> ( $\mu\text{g mg}^{-1}$  dry wt.)

ND - not determined

inner membrane and was lost during centrifugation.

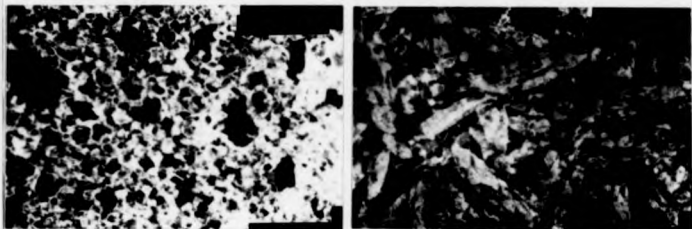
The values obtained for succinate dehydrogenase activity are given in Figure 28. The maximum activity was found in the T band, enriched approximately 3.5 fold over the cell envelope. Enzyme activity decreased down the sucrose gradient and B1 and B2 bands contained approximately 28% and 20% respectively of the activity of the T band. As discussed in section 1.2.1, succinate dehydrogenase and NAD(P)H oxidase are "markers" of the cytoplasmic membrane. Consequently, the data indicated an enrichment of cytoplasmic membrane in the T band from the first sucrose gradient.

#### 5.3.3 Electron microscope

Electron micrographs in Figure 31 illustrated the differences in the morphology of the subcellular material located in each fraction. The T band contained small vesicles of uniform size, characteristic of membrane fragments (Miura & Mizushima, 1969). B1 and B2 consisted of morphologically identical long, open tubular structures of varying sizes characteristic of the morphology of cell wall fragments (Collins & Neiderman, 1976b).

#### 5.3.4 Pigment content

The adsorption spectra from the three bands are given in Figure 32. It could be clearly seen that only the T band contained significant amounts of pigment as was expected from its red colouration. The lower bands, B1 and B2 were white in colour. The adsorption spectrum from the T band was not directly comparable to those from whole cells or extracted photopigments reported by Eckersley and Dow (1980) for the following reasons. Firstly, the growth of *Rp. blastica* was chemoheterotrophic



a

b

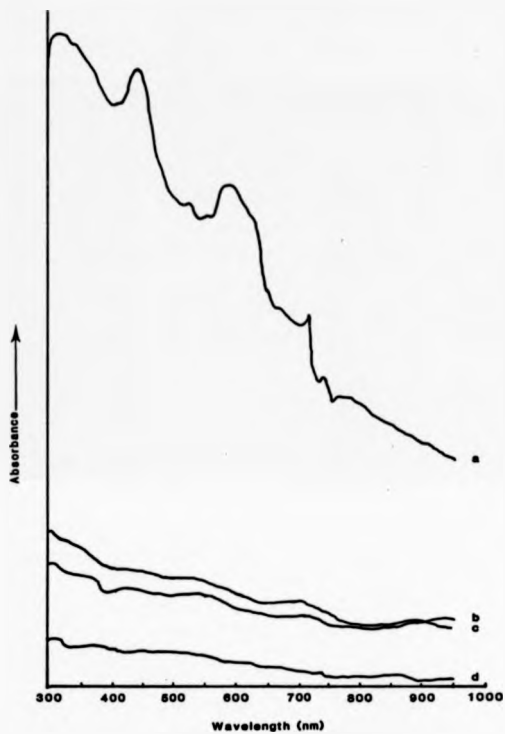
**Figure 31**      Electron micrographs of a) cytoplasmic membrane enriched  
T band; b) cell wall fraction B1

Samples were dialysed against water to remove sucrose and stained with phosphotungstic acid (x50,000). Bar represents 0.1  $\mu\text{m}$ .

Figure 32

Adsorption spectrum of cell envelope fractions isolated  
by sucrose differential density centrifugation.

Samples were diluted to  $1.5 \text{ mg ml}^{-1}$  in 60% (w/w) sucrose and scanned in  
a Pye Unicam SP8 double beam spectrophotometer 300-900 nm in quartz  
cuvettes. a) cytoplasmic membrane; b) cell wall fraction B1; c) cell  
wall fraction B2. d) 60% (w/w) sucrose.



which does not allow the synthesis of photopigments as observed with photoheterotrophic growth. Differences in the photopigment content with chemoheterotrophic and photoheterotrophic growth have been reported for *Rm. vannielii* (Kelly, 1985) and *Rb. capsulatus* (Garcia et al., 1981). Secondly, the adsorption spectrum in this study was from membrane fragments and not from whole cells or extracted photopigments.

#### 5.4 Triton Extraction of a Cell Wall Fraction

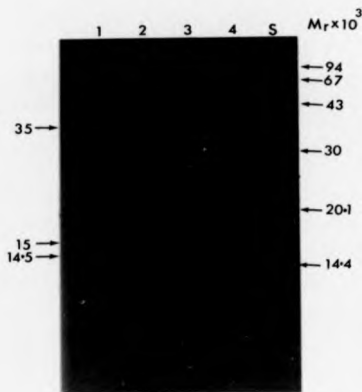
To remove contaminating membrane from cell wall fractions of *Rb. capsulatus*, Triton X-100 extraction has been shown to be required (Flammann and Weckesser, 1984a). As Triton X-100 is a non-ionic detergent in which peptidoglycan is insoluble, contaminating cytoplasmic membrane, which is soluble, is readily removed.

To determine whether the cell wall fractions from *Rp. blautica* could be further purified, fraction B2 from the second sucrose gradient was Triton extracted. The polypeptide patterns before and after extraction were observed and showed no significant differences (Fig. 33), indicating that two sucrose gradients were sufficient to purify the cell wall fractions from contaminating cytoplasmic membrane.

#### 5.5 Discussion

Data from amino acid analysis, enzymatic assays, electron microscopy and adsorption spectra identified the three bands T, B1 and B2. Each of these analyses was specific for either the cell wall or cytoplasmic membrane. The absence of peptidoglycan, increased succinate dehydrogenase activity compared to a cell envelope preparation, the





**Figure 31** Polypeptide patterns of cell envelope fractions from *Rp. blautica*

SDS-PAGE: 10% (w/v) linear gel. Lane 1, 30  $\mu$ g of cytoplasmic membrane (T band); lane 2, 30  $\mu$ g of cell wall fraction B1; lane 3, 30  $\mu$ g of cell wall fraction B2; lane 4, 30  $\mu$ g of cell wall fraction B2 after triton X-100 purification. Lane S, molecular weight standards. Samples solubilised in sample buffer at 100°C for 5 min. Gel stained with coomassie blue.

presence of photopigments and the morphology of the vesicles suggested that the T band was highly enriched for cytoplasmic membrane. The lower two bands B1 and B2 contained Mur and DAP (markers for peptidoglycan), and much reduced succinate dehydrogenase activity compared to the cell envelope preparation, no detectable photopigments and the morphology of the vesicles from electron micrographs suggested that these two fractions were of cell wall origin.

Similar protocols have been used to identify fractions after differential density sucrose gradient centrifugation of cell envelopes from other members of the *Rhodospirillaceae*, *Rb. sphaeroides* (Ding and Kaplan, 1976; Neiderman and Gibson, 1971), *Rs. rubrum* (Oelze *et al.*, 1975) and *Rb. capsulatus* (Flammann and Weckesser, 1984a). In all reports the cytoplasmic and intracytoplasmic membranes have been less dense than cell wall fractions. Only with *Rb. capsulatus* (Flammann and Weckesser, 1984a) have two cell wall fractions of different densities been reported, but unlike *Rp. blastica*, there appeared to be no difference in the amount of cross linking in the peptidoglycan of each fraction.

#### 5.6 Conclusions

- a) Gentle lysis failed to isolate the outer membrane of *Rp. blastica*.
- b) French pressing of *Rp. blastica* cells, isolation of the cell envelope and subsequent differential density sucrose gradient centrifugation separated three distinct bands.
- c) From succinate dehydrogenase activity, electron microscopy, adsorption spectra and amino acid analysis the T-fraction was identified as cytoplasmic membrane, and fractions B1 and B2 as cell wall fractions.

## 6. Chemical Analysis of the Cell Envelope Fractions

### 6.1 Chemical Analyses of the Cell Envelope Fractions

Chemical analyses of the cell envelope fractions of *Rp. blastica* were undertaken and the results compared to other members of the *Rhodospirillaceae*. Comparisons between *Rp. blastica* and *Rp. blastica*<sup>r9</sup> were made in an attempt to observe differences that may result in the loss of phage adsorption to *Rp. blastica*<sup>r9</sup>.

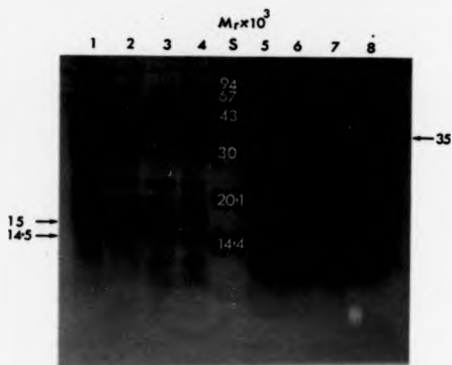
#### 6.1.1 Analysis of polypeptide patterns

Considerable work has been undertaken on the polypeptide patterns of cell envelope fractions of the *Rhodospirillaceae* and in particular *Rb. sphaeroides* and *Rb. capsulatus* (section 1.2.3). As previously discussed in section 1.3.4.1 one of the major classes of phage receptors are proteins, therefore comparisons between the polypeptide patterns from *Rp. blastica* and *Rp. blastica*<sup>r9</sup> cell envelope fractions were undertaken.

##### 6.1.1a Polypeptide patterns from *Rp. blastica*

Figure 34 shows the polypeptide patterns obtained after SDS-PAGE of enriched cytoplasmic membrane and cell wall fractions B1 and B2 from *Rp. blastica*. The corresponding densitometer scans are shown in Figure 35. It was obvious from Figure 34 that each fraction had its own distinct complex polypeptide banding pattern and only shared some minor bands.

After solubilisation at 100°C, the two cell wall fractions B1 and B2 revealed a common major protein of Mr 35,000. This Mr 35,000 protein was found to be heat modifiable in that distinct changes occurred when solubilisation conditions were altered (Fig. 36), suggesting a possible peptidoglycan association. At temperatures below 70°C only trace



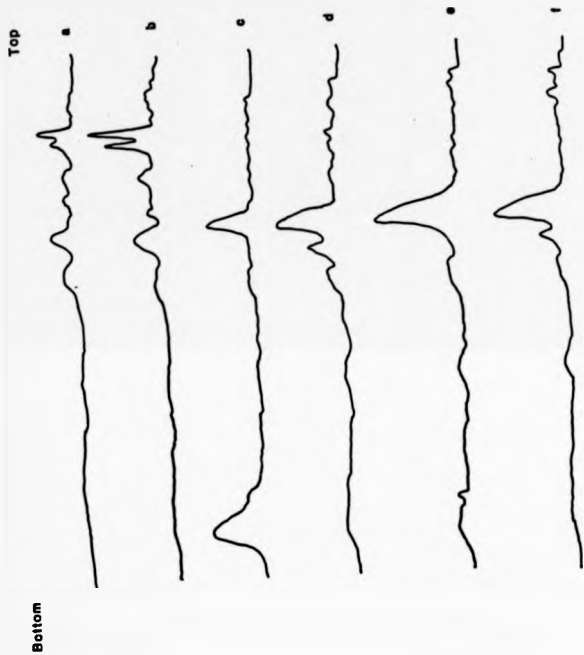
**Figure 34** Polypeptide pattern of cell envelope fractions of  
*Rp. blastica* and *Rp. blastica*<sup>r9</sup>

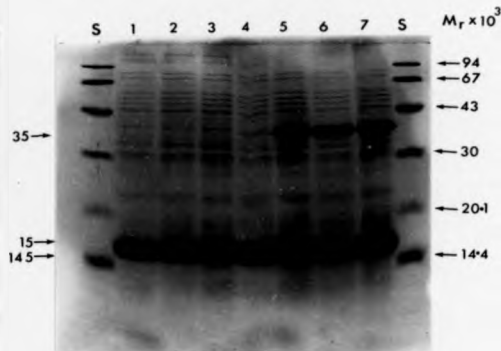
SDS-PAGE: 10% (w/v) polyacrylamide. 30  $\mu$ g of protein, solubilised in sample buffer at 100°C for 5 min. Lane 1, cytoplasmic membrane *Rp. blastica*; lane 2, cell wall B1 *Rp. blastica*; lane 3, cell wall B2 *Rp. blastica*; lane 4, triton X-100 insoluble cell wall B2 *Rp. blastica*; lane 5, cytoplasmic membrane *Rp. blastica*<sup>r9</sup>; lane 6, cell wall B1 *Rp. blastica*<sup>r9</sup>; lane 7, cell wall B2 *Rp. blastica*<sup>r9</sup>; lane 8, triton X-100 insoluble cell wall B2 *Rp. blastica*<sup>r9</sup>; lane 9, molecular weight standard proteins.

Figure 35

Densitometer scans after SDS-PAGE of cell envelope fractions from *Rp. blastica* and *Rp. blastica*<sup>r9</sup> shown in Figure 34

a) cytoplasmic membrane of *Rp. blastica*; b) cytoplasmic membrane of *Rp. blastica*<sup>r9</sup>; c) cell wall fraction B1 of *Rp. blastica*; d) cell wall fraction B1 of *Rp. blastica*<sup>r9</sup>; e) cell wall fraction B2 of *Rp. blastica*; f) cell wall fraction B2 of *Rp. blastica*<sup>r9</sup>.





**Figure 36** Influence of the solubilisation temperature on the cell wall B1 polypeptide pattern of *Rp. blastica*

SDS-PAGE: 10% (w/v) polyacrylamide. Samples were solubilised in sample buffer at various temperatures for 5 or 20 min; 30  $\mu$ g of each protein was applied to the gel. Lane 1, 55°C 20 min; lane 2, 60°C 20 min; lane 3, 65°C 20 min; lane 4, 70°C 20 min; lane 5, 75°C 20 min; lane 6, 80°C 20 min; lane 7, 100°C 5 min; lane S, molecular weight standard proteins.

amounts of the Mr 35,000 protein were detectable, but at 75°C and above this protein could be clearly seen as one of the dominant cell wall proteins. The polypeptide pattern from cell walls of other phototrophic bacteria investigated to date also contain dominant proteins of similar molecular weights. They were shown to be peptidoglycan associated in *Rb. capsulatus* (Mr 33,000) (Flammann and Weckesser, 1984a), *Rb. sphaeroides* (Mr 47,000) (Deal and Kaplan, 1983) but not in *Chromatium vinosum* (Mr 42,000) (Lane and Hulbert, 1980). The proteins from *Rb. capsulatus* and *Rb. sphaeroides* have a porin activity in the phospholipid liposome swelling assay (Flammann and Weckesser, 1984b; Weckesser *et al.*, 1984). The heat modifiable nature of the Mr 35,000 protein from *Rp. blastica* may indicate that it too has a possible porin function.

In addition to the Mr 35,000 protein, cell wall fraction B1 contained two unique proteins, a Mr 15,000 protein which could occasionally be resolved from a more intense Mr 14,500 protein (Fig. 34). Neither appeared to be heat modifiable. *Rp. blastica* is therefore unusual amongst the other photosynthetic bacteria in that it has three dominant cell wall proteins. More important is the specific segregation of the Mr 15,000 and 14,500 proteins into the cell wall fraction B1. Flammann and Weckesser (1984a) reported no such segregation of proteins between the two cell wall fractions isolated from *Rp. capsulatus*. Such segregation of proteins between cell wall fractions and data from the amino acid analysis (section 5.3.1) which indicated that B1 has less peptidoglycan cross linkage than B2, suggest that the two fractions B1 and B2 do indeed represent distinct topographical fractions of the cell. For example, in *Rp. blastica* they could represent the longitudinal walls of the cell, i.e. the lateral cell walls, and the polar (cap) region of the cell.



Studies on the segregation of proteins have been undertaken in *Caulobacter crescentus*. Methyl-accepting chemotaxis proteins (MCPs) are only synthesised in the predivisive cell concomitant with the biosynthesis of the polar flagellum (Nathan *et al.*, 1986). Upon division both the flagellum and the MCPs are partitioned to only one daughter cell, the swarmer cell. Penicillin-binding proteins (PBPs) in isolated stalks were found to be different from those of complete cell envelopes (Koyasu *et al.*, 1983). The problem of how these proteins are targeted to distinct membrane sites is still unanswered.

#### 6.1.1b Comparisons between *Rp. blasticus* and *Rp. blasticus*<sup>r9</sup>

No detectable differences in the polypeptide pattern of the cytoplasmic membrane from *Rp. blasticus* and *Rp. blasticus*<sup>r9</sup> were observed (Figs. 34 and 35). However, differences were detected between the cell wall fractions B1 and B2. In *Rp. blasticus*<sup>r9</sup> there was an apparent increase in the relative intensity of two polypeptides of Mr 32,000 and 33,000. In densitometer scans (Fig. 35) these appeared as a single combined peak, these proteins could not be detected in *Rp. blasticus*. These differences in intensity were emphasised by uneven loading of the samples on gels, presumably caused by the inherent variability between repeated protein assays. However, the major difference in Figure 34 was the virtual absence in *Rp. blasticus*<sup>r9</sup> of the Mr 14,500 and 15,000 proteins specifically located in cell wall fraction B1 in *Rp. blasticus*, this being confirmed in the densitometric scans (Fig. 35).

#### 6.2 Analysis of lipopolysaccharide

Various domains of the lipopolysaccharide molecule are receptors for many phage (section 1.3.4). To determine if LPS was involved in the

adsorption of ØBHG1, chemical analysis of *Rp. blastica* and *Rp. blastica*<sup>r9</sup> LPS were undertaken, thereby enabling identification of putative components of phage adsorption.

#### 6.2.1 Size distribution analysis of *Rp. blastica* lipopolysaccharide LPS

Size distribution analysis of the polysaccharide moiety from *Rp. blastica* LPS (WPH) is shown in Figure 37. There was a wide range of sizes obtained from Mr  $9.4 \times 10^2$  to  $7 \times 10^5$  (Table 8). The peak at 12.946 min was the sharpest and largest component which corresponded to a value of Mr  $9.7 \times 10^4$ .

The use of size exclusion chromatography is relatively new, especially where linear polymers are used as molecular weight standards. Previous methods employed to study size distribution of polysaccharides included fractionation from DEAE-cellulose columns and SDS-PAGE (Sutherland, 1977). Much of the data on size heterogeneity of the polysaccharide moiety in LPS is related to *Salmonella* species where LPS preparations represent a family of molecules differing in chain length and with lipid A molecules carrying no side chains (Wilkinson, 1977). From Figure 37 and Table 9 the 20.765 min peak (Mr 940) probably corresponded to unsubstituted cores, while the 17.688 min (Mr  $7.1 \times 10^3$ ) and 12.948 min (Mr  $9.7 \times 10^4$ ) peaks corresponded to partial and complete O antigens attached to cores. The higher molecular weight peaks at 11.543 and 10.2 min corresponding to Mr  $3.4 \times 10^5$  and  $7 \times 10^5$  respectively, indicated the presence of larger chains of polysaccharides suggesting the presence of exopolysaccharides (EPS).



Figure 37      Size exclusion chromatography of lipid-free  
LPS (WPH) from *Ep. blastica*

Sample analysed on a TSK400 SEC column, water eluant  $0.5 \text{ ml min}^{-1}$ ,  
refractive index detection. Pullulan standards for molecular weight  
markers.

Table 8

Molecular weights of peaks obtained from size exclusion chromatography of LPS (WPH) *Rp. blasticus* using Pulluman standards as molecular weight markers

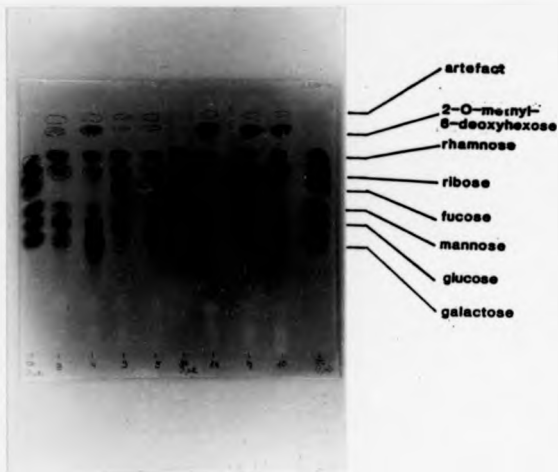
Time (min)	Molecular Weight (daltons)
10.2	$7 \times 10^5$
11.543	$3.4 \times 10^5$
12.948	$9.7 \times 10^4$
17.688	$7.1 \times 10^3$
20.765	$9.4 \times 10^2$

6.2.2 Thin layer chromatography of *Rp. blastica* and *Rp. blastica*<sup>r9</sup>  
lipopolysaccharide (LPS)

Samples of purified LPS and material from the supernatant of LPS washes from *Rp. blastica* and *Rp. blastica*<sup>r9</sup> were hydrolysed then analysed by thin layer chromatography. The results are presented in Figures 38 and 39.

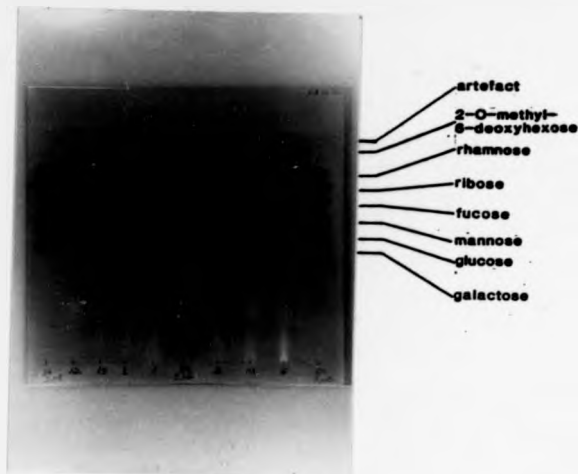
The neutral sugars found in *Rp. blastica* were as described by Tegtmeier *et al.* (1985). These were galactose, glucose, mannose, rhamnose and the unusual neutral sugar 2-O-methyl-6-deoxyhexose (MDH). However, in addition ribose was also present in this study. Ribose is often a contaminant sugar associated with RNA present due to incomplete washing. During purification, LPS was washed three times to remove any such contaminants, and when samples were further treated with RNAase and run on agarose gels along side untreated samples no differences were observed, indicating no gross contamination of the LPS with high molecular weight RNA. The results suggested that ribose was a true constituent of *Rp. blastica* LPS. It should be noted that the intensity of individual spots on TLC plates was not necessarily quantitative as some sugars stain more intensively than others. However, any relative increase or decrease in the proportion of sugars present could be studied.

On comparison of LPS isolated from the water phase (WPH) and the phenol phase (PHPH) of *Rp. blastica* there was a relative increase in the intensity of glucose and galactose in the PHPH. Free core units of LPS are characteristically hydrophobic and would therefore be preferentially extracted into the PHPH. This would suggest that galactose and glucose were associated with the core unit, as with glucosamine which could be



**Figure 31** TLC analysis of samples from various stages of LPS preparation of *Rp. blasticus* and *Rp. blasticus*<sup>T9</sup>

Approximately 1 mg (dry weight) of samples were hydrolysed (4 hr 100°C, 0.5 M H<sub>2</sub>SO<sub>4</sub> followed by neutralisation with saturated Ba(OH)<sub>2</sub>) and run on cellulose glass plates; solvent system: n-1-butanol:pyridine:water (6:4:3 vol). Stained with anilinium hydrogen phthalate. Lane 9, LPS (WPH) *Rp. blasticus*; lane 4, LPS (PHPH) *Rp. blasticus*; lane 3, LPS (WPH) *Rp. blasticus*<sup>T9</sup>; lane 5, LPS (PHPH) *Rp. blasticus*<sup>T9</sup>; lane 14, supernatant I LPS (WPH) *Rp. blasticus*; lane 7, supernatant II LPS (WPH) *Rp. blasticus*; lane 10, supernatant III LPS (WPH) *Rp. blasticus*; Lane 11, standard sugars.



**Figure 12** TLC plate of samples from various stages of LPS preparation of *Ep. blastica* and *Ep. blastica*<sup>r9</sup>

For conditions see Figure 18. Lane 12, supernatant I LPS (WPH) *Ep. blastica*<sup>r9</sup>; lane 13, supernatant II LPS (WPH) *Ep. blastica*<sup>r9</sup>; lane 8, supernatant III LPS (WPH) *Ep. blastica*<sup>r9</sup>; lane 1, supernatant I LPS (PHPH) *Ep. blastica*; lane 2, supernatant II LPS (PHPH) *Ep. blastica*; lane 11, supernatant I LPS (PHPH) *Ep. blastica*<sup>r9</sup>; lane 6, supernatant II LPS (PHPH) *Ep. blastica*<sup>r9</sup>; Lane St, standard sugars.

observed in lipid A-free polysaccharide (Tegtmeier *et al.*, 1985). A considerable amount of the other neutral sugars was observed in the PHPH, but more noticeable was the amount of sugars detected in the washes of both the WPH and PHPH LPS. The relative increase in ribose found in the supernatants reflected the contaminating RNA as described above, but the other neutral sugars in such quantities may have indicated that EPS was present. This was further supported by the size distribution analysis (section 6.2.1) which showed the presence of a range of high molecular weight polysaccharides. The presence of neutral sugars in the supernatant from washes of the LPS from both *Rp. blastica* and *Rp. blastica*<sup>r9</sup> is in contradiction to results obtained by Tegtmeier *et al.* (1985) who did not find any such free sugars.

#### 6.2.3 Quantitative analysis of the neutral sugars

Several problems were encountered during quantitative analysis of the neutral sugar content of cell envelope fractions and LPS. Figure 40 shows a GLC trace of a cell wall sample from *Rp. blastica*, the MDH sugar appeared only in trace amounts.

The alditol acetate derivative of this MDH sugar formed for GLC analysis was highly volatile and readily lost during rotary evaporation of the samples. Thus comparisons of this sugar between cell wall fractions and strains was not possible using this technique.

Table 9 confirmed a reduction in the galactose content of both cell wall fractions from *Rp. blastica*<sup>r9</sup> as seen in the TLC analysis.

Interpretation of the data was made more difficult because of the low sugar content of cell walls. It must be assumed that the ratios of sugars found were true values, but it must be noted that it is very



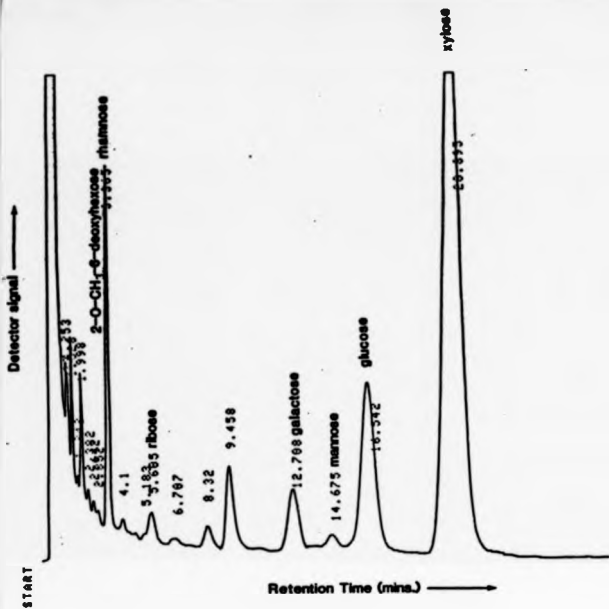


Figure 60

GLC trace of alditol acetate derivatives of the neutral  
sugars of cell wall fraction E2 from *Rp. blasticus*

Glass column (150 cm x 0.3 cm Ø) packed with ECNSS-M. Column  
temperature 180°C. Carrier gas N<sub>2</sub> (30 ml/min).

Table 9

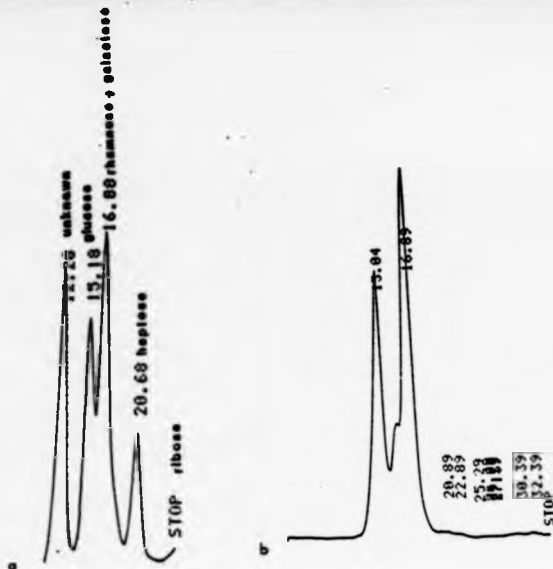
Neutral sugar content ( $\mu\text{g mg}^{-1}$  dry wt.) of various cell envelope fractions from *Rp. blastica* and *Rp. blastica*<sup>r9</sup> determined by gas liquid chromatography

Neutral Sugar	<i>Rp. blastica</i>			<i>Rp. blastica</i> <sup>r9</sup>	
	cytoplasmic membrane	cell wall B1	cell wall B2	cell wall B1	cell wall B2
Rhamnose	1.431	2.126	2.034	1.902	1.5
Ribose	0.239	0.423	0.101	0.216	0.215
Arabinose	0	0	0	0	0
Xylose	0	0	0	0	0
Mannose	1.163	1.747	2.525	1.071	3.054
Galactose	0.446	0.169	0.315	0.045	0.060
Glucose	4.486	7.196	6.103	6.337	7.140

difficult to prove that differences in the other sugars were not due to contamination by EPS possibly associated with *Rp. blastica* cells.

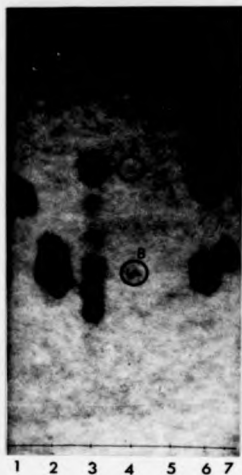
The cytoplasmic membrane should not contain any detectable amounts of the neutral sugars found in the LPS and EPS since it is a simple protein phospholipid bilayer. However, when analysed, neutral sugars were detected (Table 9). This suggested that contaminating LPS and/or EPS was present throughout the sucrose gradient.

In an attempt to overcome the problems associated with the volatile derivative of MDH, neutral sugars were also measured by HPLC (Sutherland and Kennedy, 1986). An HPLC trace (Fig. 41a) of *Rp. blastica* LPS (WPH) suggested that the 12.28 min peak corresponded to the MDH sugar as 2-O-methylglucose and 3-O-methylxylose have retention times of 15.43 and 14.59 min respectively (Sutherland and Kennedy, 1986). However, when samples of LPS from *Rp. blastica*<sup>r9</sup> were analysed the 12.28 min peak was still present and at approximately the same quantity (Fig. 41b). This suggested that either the intensity change of the MDH sugar observed by TLC (section 6.2.2) was an artifact or that the 12.28 min peak did not correspond to the methyl sugar. A paper chromatograph of various samples described below was run and stained with silver nitrate (Fig. 42). Lipid A free LPS (WPH) from *Rp. blastica* contained all the sugars, i.e. rhamnose, glucose, galactose, mannose, MDH and glucosamine, whilst the 16.8 min peak from the HPLC analysis contained trace amounts of rhamnose, glucose and galactose. As expected, a concentrate of six pooled 12.28 min peaks showed no sugar, suggesting that the peak was an artifact of the hydrolysis process. This shows the importance of known sugar standards in such a novel system. Problems were also encountered with the measurement of galactose as this sugar appeared in a combined



**Figure 41** HPLC traces of LPS isolated from *Rp. blasticus* and *Rp. blasticus*<sup>r9</sup>

Lipid A free LPS, run on a Brownlee Pb<sup>2+</sup> cartridge, water as eluent at 80°C, 0.2 ml min<sup>-1</sup>, refractive index detection. Trace a), LPS (UPH) *Rp. blasticus*; Trace b) LPS (UPH) *Rp. blasticus*<sup>r9</sup>. Elution time: 12.28, 13.04 min, unknown; 15.18 min, glucose, 16.88, 16.89, rhamnose and galactose; 20.68, 20.89 min, heptose.



**Figure 42**

Descending paper chromatography to determine the nature of the 12.28 min peak observed in HPLC analysis of Lipid A-free LPS from *Rp. blastica* and *Rp. blastica*<sup>r9</sup>.

Solvent system n-1-butanol:pyridine:water (6:4:3) for 36 hrs.

Lane 1, ribose standard; lane 2, mannose and glucose standards; lane 3, hydrolysed lipid A-free LPS (WPH) *Rp. blastica*; lane 4, 16.8 min eluant peak from HPLC; lane 5, 12 min eluant peak from HPLC; lane 6, rhamnose and glucose standards. Lane 7, fructose standard . A, rhamnose; B, mannose.

peak with rhamnose. Further separation on a second column was required but attempts to separate these sugars using an amino silicate column were unsuccessful due to the fluctuating base line, associated with this column.

#### 6.2.4 Comparison of neutral sugars of lipopolysaccharide (LPS) from *Rp. blastica* and *Rb. sphaeroides* 8253

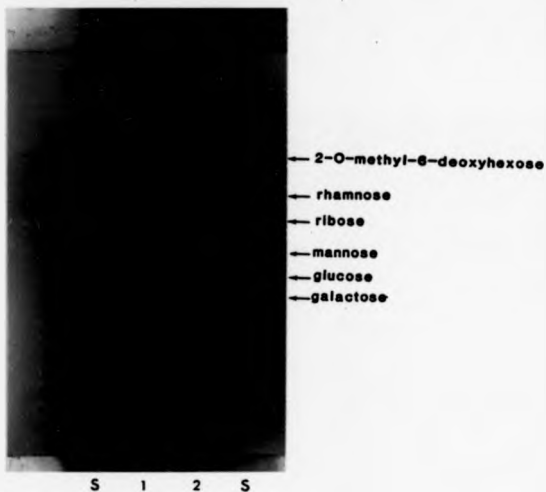
The neutral sugars from LPS of *Rp. blastica* and *Rb. sphaeroides* 8253 were compared by TLC (Fig. 43). *Rb. sphaeroides* 8253 contained rhamnose, ribose, mannose, galactose and glucose as did *Rp. blastica* (section 6.2.2), but no MDH could be detected and mannose appeared to be present in a relatively higher concentration.

This similarity of LPS subunits would help to explain the transient adsorption and 'lysis from without' of *Rb. sphaeroides* 8253 by ØBHG1 whereby it could associate with cell wall components similar to those of its natural host.

#### 6.3 Fatty Acid Analysis of Cell Envelope Fractions

Fatty acids were analysed after transesterification to their methyl ester derivatives by GLC. A typical trace is shown in Figure 44 and the fatty acid content of the various cell wall fractions are given in Table 10.

Tegtmeyer *et al.* (1985) characterised the fatty acid of LPS isolated from *Rp. blastica*. From this it was apparent that the C10-0-3-OH, C10-0-OCCH<sub>3</sub>-3, and C14-0 fatty acids associated with LPS were not resolved by the GLC analysis in this study. These short chain fatty



**Figure 43** Thin layer chromatograph of hydrolysed LPS from *Rp. blastica* and *Rh. sphaeroides* 8253

Approximately 1 mg (dry weight) of hydrolysed LPS (4 hr 100°C, 0.5 M  $H_2SO_4$ , followed by neutralisation by  $Ba(OH)_2$ ), run on a cellulose glass plate. Solvent system: n-1-butanol:pyridine:water (6:4:3 vol). Stained with anilinium hydrogen phthalate. Lane 1, *Rp. blastica* LPS (UPH); lane 2, *Rh. sphaeroides* 8253 LPS. Lane 3, standard sugars 2 µg.

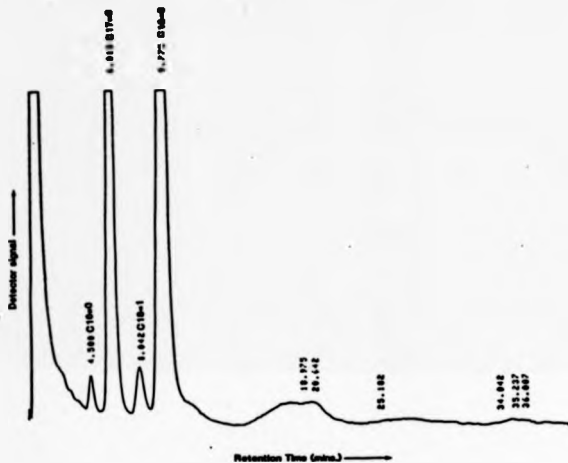


Figure 6a

Gas-liquid chromatograph of fatty acids of cell wall fraction B1 from *Rp. blasticus* after transesterification to their methyl ester derivatives (section 2.18.1).



Table 10

Fatty acid content of various cell envelope fractions from *Rp. blasticar* and  
*Rp. blasticar*<sup>9</sup> ( $\mu\text{g mg}^{-1}$  dry wt)

Fatty acids	<i>Rp. blasticar</i>				<i>Rp. blasticar</i> <sup>9</sup>			
	cytoplasmic membrane	cell wall B1	cell wall B2	Triton cell wall B2	cytoplasmic membrane	cell wall B1	cell wall B2	Triton cell wall B2
C14:0-3-oxo	3.1	3.3	3.1	0.3	3.7	1.5	2.5	0.4
C16:0	9.2	2.5	2.2	1.1	8.1	3.0	5.0	2.3
C18:0	19.3	3.4	4.1	5.7	19.8	4.9	6.3	2.3
C18:1	81.7	36.5	34.1	15.3	83.2	29.5	34.5	23.4

acids could only be detected on long capillary columns which increase their retention times and resolution from the solvent peak. However, the longer chain fatty acids were detected and included C14=0-3-Oxo, C16=0, C18=0 and C18=1.

From Table 10 no significant differences were apparent in the fatty acid contents of *Rp. blastica* and *Rp. blastica*<sup>r9</sup>. There also appeared to be no segregation of fatty acids between cell wall fractions B1 and B2. Fatty acid C18=1 was dominant. The cytoplasmic membrane fraction showed a considerably higher concentration of C18=1 (30 µg/mg dry weight) when compared to the cell wall fractions. Phototrophic bacteria in general are noted for the high content of unsaturated fatty acids in their cytoplasmic membranes (Gelze and Drews, 1972). There also appeared to be a slight increase in the concentration of C16=0 and C18=0 fatty acids in the cytoplasmic membrane in agreement with results obtained for *Rb. capsulatus* (Flammann and Weckesser, 1984a).

After Triton X-100 extraction of cell wall fraction B2 in the presence of magnesium, there was a general decrease in the concentration of each of the fatty acids, and in particular the C18=0 and C18=1. Similar decreases have been observed after Triton X-100 extraction of cell walls from *Rb. capsulatus* (Flammann and Weckesser, 1984a).

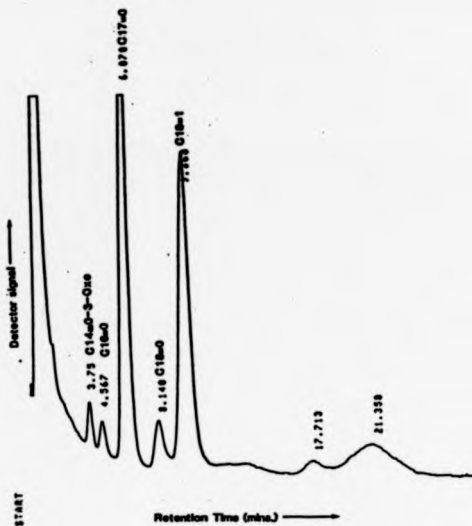
#### 6.4 Peptidoglycan Analysis

Peptidoglycan isolated from *Rp. blastica* was analysed for the presence of neutral sugars and fatty acids. No neutral sugars were detected indicating that LPS and therefore the outer membrane had been removed. Cytoplasmic membrane would also have been reported after boiling in SDS.

Figure 45 shows the GLC trace obtained after fatty acid analysis of the peptidoglycan. Considerable quantities of fatty acids remained indicating that lipoproteins were present. These proteins, as discussed previously (section 1.2.5), are bound to the peptidoglycan layer, and these bonds are not broken by boiling in SDS. However, the proteins and phospholipids bound by electrostatic bonds are readily removed. Further studies on peptidoglycan could include treatment by specific proteolytic enzymes and the subsequent detection of cleaved proteins by SDS-PAGE.

#### 6.5 Conclusions

- a) Each cell envelope fraction had its own unique polypeptide pattern. Cell wall fractions were dominated by a heat modifiable Mr 35,000 protein and Mr 14,500 and 15,000 proteins specifically located in cell wall fraction B1.
- b) The Mr 14,500 and 15,000 proteins appeared to be absent in *Rp. blastica*<sup>r9</sup>
- c) Size distribution and TLC analysis of LPS suggested the presence of EPS.
- d) *Rp. blastica*<sup>r9</sup> LPS appeared to show a decrease in galactose and almost complete absence of 2-O-methyl-6-deoxyhexose.
- e) *Rb. sphaeroides* 8253 contained similar sugars to those of *Rp. blastica* except no 2-O-methyl-6-deoxyhexose was detected.
- f) No significant differences were detected in the fatty acids of *Rp. blastica* and *Rp. blastica*<sup>r9</sup>. The cytoplasmic membrane contained increased amounts of C18-0 and C18-1 compared to the cell wall fractions B1 and B2.
- g) The presence of fatty acids in peptidoglycan isolated from *Rp. blastica* indicated the presence of lipoproteins.



**Figure 45**

Gas-liquid chromatograph of fatty acids of peptidoglycan from *Rp. blastica* after transesterification to their methyl ester derivatives (section 2.18.1)

## 6.6 Identification of the Phage Receptor for $\phi$ BHGl

### 6.6.1 Inactivation of $\phi$ BHGl with purified lipopolysaccharide LPS

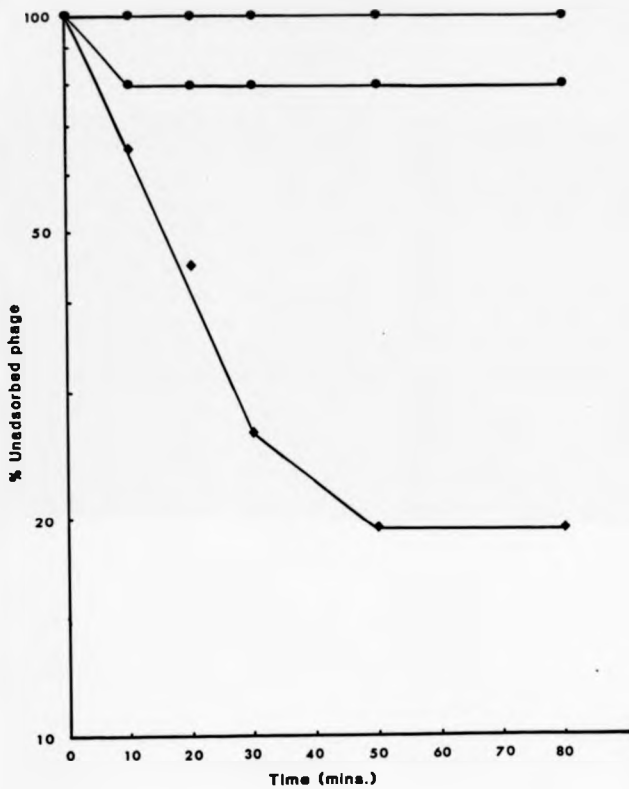
The rate of inactivation of  $\phi$ BHGl with purified LPS isolated from *Rp. blastica* and *Rp. blastica*<sup>r<sub>9</sub></sup> is shown in Fig. 46. After 50 min approximately 80% of phage were inactivated by LPS (WPH). A concentration of 1.15 mg ml<sup>-1</sup> of LPS was found to inactivate  $\phi$ BHGl at a suitable rate, however, a ten-fold dilution resulted in no detectable inactivation of phage. LPS (WPH) isolated from *Rp. blastica*<sup>r<sub>9</sub></sup> did not inactivate  $\phi$ BHGl in these assays. This suggests that an essential component(s) found in the LPS from *Rp. blastica* was required for the adsorption of  $\phi$ BHGl and that it was absent in LPS from *Rp. blastica*<sup>r<sub>9</sub></sup>. As described in section 6.2.2 there were two apparent differences in the neutral sugar content of the resistant strain, a reduction in the concentration of galactose and virtual loss of 2-O-methyl-6-deoxyhexose (MDH) suggesting that one or both may be required for phage adsorption.

The concentration of LPS (WPH) (1.15 mg ml<sup>-1</sup>) used for the phage inactivation experiments was relatively high when compared to that reported for other phage (Tomás and Jofre, 1985; Samimi and Drews, 1978). Solutions of LPS may be sonicated to break up vesicles formed on resuspension. Similar treatment of the LPS from *Rp. blastica* drastically reduced the percentage of  $\phi$ BHGl inactivated from 80% to 20% (Fig. 46). An explanation for this observation would be a requirement for an LPS complex which is broken down into smaller molecular fragments (upon sonication). A similar result was observed with phage FO when inactivated with LPS isolated from *Salmonella*. Lindberg, (1973), indicated that a complex with a molecular weight of more than  $9 \times 10^4$  was required for irreversible attachment of the FO phage.

Figure 46

Inactivation of  $\phi$ BHCl by LPS from *Rp. blastica*  
and *Rp. blastica*<sup>r9</sup>

$10^5$  pfu was added to 1.15 mg LPS resuspended in 1 ml PBS and incubated at 30°C. Samples were removed at varying times to assay for active phage. ◆---◆, LPS (WPH) *Rp. blastica*; ●---●, LPS (WPH) *Rp. blastica* sonicated before the addition of phage; ○---○, LPS (WPH) *Rp. blastica* pronase treated; ▼---▼ LPS (WPH) *Rp. blastica*<sup>r9</sup>.  $10^5$  pfu ml<sup>-1</sup> of  $\phi$ BHCl in PBS showed no decrease in plaquing activity.



Inactivation of ØBHG1 may also have been due to protein contamination in the LPS (WPH) from *Rp. blastica*. The method of extraction employed (Westphal *et al.*, 1952) is noted for its efficiency at removing protein. However, as a confirmatory check LPS from both the phenol and water phases from both strains were subjected to amino acid analysis the results of which are presented in Table 11.

These results indicated that extraction of LPS by this procedure did result in significant contamination by amino acids or proteins. As described above, ØBHG1 required high concentrations of LPS for inactivation and thus an involvement of contaminating proteins cannot be eliminated. In an attempt to remove any such proteins, LPS (WPH) from *Rp. blastica* was treated with pronase then used to inactivate ØBHG1. The pronase treated LPS lost its ability to inactivate ØBHG1 (Fig. 46). Assuming that this treatment did not alter the LPS in any way this result suggested that either a contaminating protein was directly involved as a phage receptor or was required for orientation of the LPS to allow inactivation of ØBHG1.

#### 6.6.2 Pre-heat treatment of *Rp. blastica*

Cultures of *Rp. blastica* were raised to a variety of temperatures before adsorption experiments with ØBHG1 were carried out at 30°C. There was no change in the rate of ØBHG1 adsorption to cells heated at 30°C or 40°C, and a decrease of only 8% to cells heated at 60°C (Fig. 47).

This heat treatment should slightly alter the arrangement of proteins within the cell wall and subsequently affect the rate of bacteriophage adsorption - should a protein receptor be involved. Such an effect has been observed with *Anacyctes nidulans* (Samimi and Drews, 1978), where



Table 11

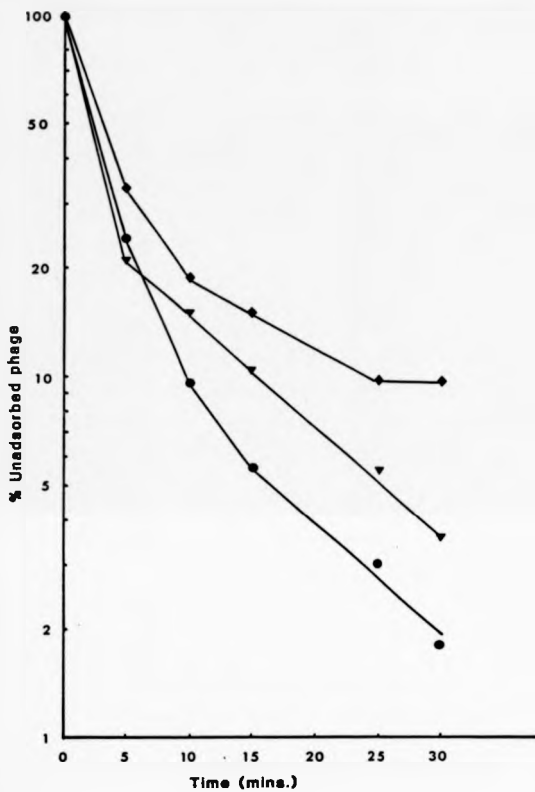
Amino acid analysis of LPS isolated from *Rp. blastica* and *Rp. blastica*<sup>r9</sup>. LPS isolated from the water and phenol phases by the Westphal (1952) procedure were subjected to amino acid analysis as described in section 2.19.3.

Strain	Phase	μ Amino acid
<i>Rp. blastica</i>	water	6.5
<i>Rp. blastica</i> <sup>r9</sup>	water	6.7
<i>Rp. blastica</i>	phenol	14.3
<i>Rp. blastica</i> <sup>r9</sup>	water <i>phenol?</i>	14.7

Figure 47

Effects of pre-heat treatment of *Rp. blastic* cells on the rate of irreversible adsorption of ØBHG1

$5 \times 10^8$  cells  $\text{ml}^{-1}$  in PBS were heated at varying temperatures for 1 hr and then equilibrated to  $30^\circ\text{C}$ .  $10^5$  pfu  $\text{ml}^{-1}$  of ØBHG1 was added and the rate of irreversible adsorption followed. ◆---◆,  $60^\circ\text{C}$ ; ▼---▼,  $40^\circ\text{C}$ ; ●---●,  $30^\circ\text{C}$ .  $10^5$  pfu  $\text{ml}^{-1}$  ØBHG1 in 0.01 M Tris-HCl, pH 6.8, showed no decrease in activity.



the adsorption of phage AS-1 was reduced by 50% after the cells were heated at 60°C for 1 hr.

From the above data, it would appear that no protein receptor is directly involved in phage adsorption.

#### 6.6.3 Pronase digestion of *Rp. blastica*

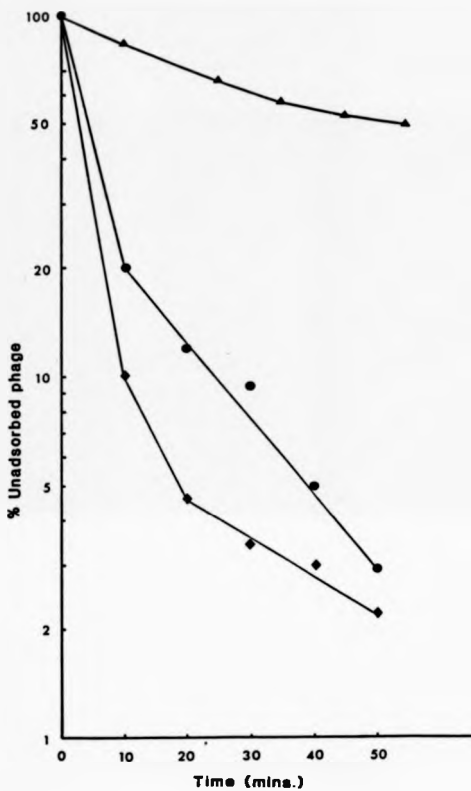
Digestion of *Rp. blastica* with pronase, a non-specific proteolytic enzyme, appeared to have no effect on the subsequent rate of adsorption of ØBHG1 (Fig. 48). To ensure that phage inactivation was not a consequence of pronase digestion by residual enzyme, present after washing the cells, a control was set up. ØBHG1 was digested with 2 µg ml<sup>-1</sup> pronase, assumed to be the residual concentration left on the cell surface after washing (Samimi and Drews, 1978). This concentration of enzyme inactivated only 5% of ØBHG1 after 1 hr incubation at 30°C. Therefore, it is clear that the decrease in free phage was due to adsorption of ØBHG1 to *Rp. blastica*.

Had a cell wall protein exposed on the cell surface of the bacterium been involved in ØBHG1 adsorption, pronase digestion should have removed this protein and prevented ØBHG1 adsorption. Samimi and Drews (1978) found a reduction of 80% in the adsorption of AS-1 to *A. nidulans* following pronase digestion. As no such decrease was observed with *Rp. blastica* this suggested that no protein was involved directly as a receptor for ØBHG1. However, when interpreting such data, it must be noted that if 'masked' by LPS or EPS a protein will be protected from enzyme digestion. As discussed in section 6.2.2, *Rp. blastica* contains LPS and also perhaps EPS both of which may protect any cell wall protein with possible phage adsorption functions.

Figure 48

Effects of pronase treatment of *Rp. blastica* on the rate of irreversible adsorption of ØBHCl.

$5 \times 10^8$  cells  $\text{ml}^{-1}$  of *Rp. blastica* were treated with  $250 \mu\text{g ml}^{-1}$  of pronase for 30 min at  $30^\circ\text{C}$  followed by washing in 0.01 M Tris-HCl, pH 6.8. The cell concentration was adjusted to  $5 \times 10^8$  cells  $\text{ml}^{-1}$  and  $10^5$  pfu  $\text{ml}^{-1}$  of ØBHCl added, the rate of irreversible adsorption was followed at  $30^\circ\text{C}$ . ●---●, pronased cells; ◆---◆, untreated cells; ▲---▲,  $10^5$  pfu  $\text{ml}^{-1}$  phage in 0.01 M Tris-HCl, pH 6.8 containing  $2 \mu\text{g ml}^{-1}$  of pronase.  $10^5$  pfu  $\text{ml}^{-1}$  of ØBHCl in 0.01 M Tris-HCl, pH 6.8, showed no decrease in plaquing activity.



6.6.4 Inactivation of ØBHG1 by cell wall and cytoplasmic membrane fractions

The rates of inactivation of ØBHG1 by the various sucrose gradient purified cell envelope fractions are shown in Fig. 49. ØBHG1 failed to be inactivated by cell envelope fractions from *Rp. blasticæ*<sup>r9</sup>, however, phage were inactivated by the two cell wall fractions B1 and B2 from *Rp. blasticæ* at an identical rate, with approximately 80% of phage activity being lost after 80 min. The cytoplasmic membrane fraction (T) inactivated only 6% of the phage in an equivalent time.

The inactivation by cell wall fractions B1 and B2 was presumably due to the ØBHG1 receptor activity associated with these fractions. The limited inactivation of ØBHG1 by cytoplasmic membrane could be explained by the presence of neutral sugars in the form of LPS and/or EPS as detected by GLC (section 6.2.3), LPS having been previously shown to be capable of inactivation of ØBHG1 (section 6.6.1). Similar observations were made with ICM from *Rp. palustris* which inactivated ØRpl (Fraund-Molbert *et al.*, 1968). This too was attributed to contaminating LPS (Bosecker *et al.*, 1972b).

6.6.5 Identification of proteins on the cell surface of *Rp. blasticæ*

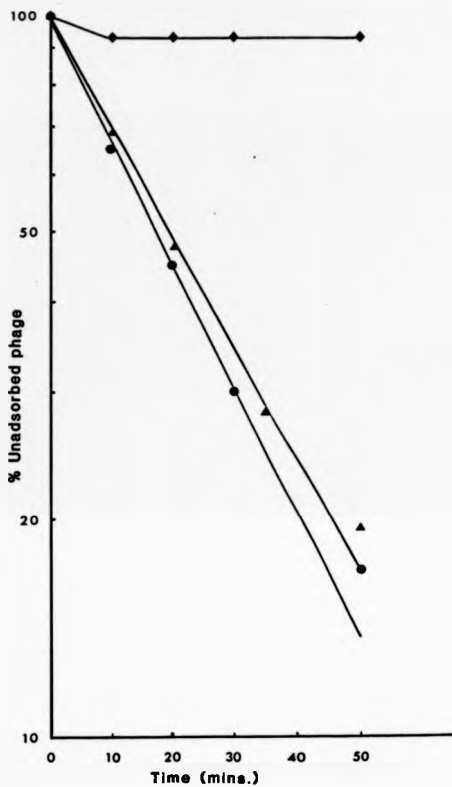
Antibodies were raised against whole cells of *Rp. blasticæ* in an attempt to identify proteins which were exposed on the cell surface. Western blots revealed only the Mr 35,000 protein from the cell wall fractions B1 and B2 was exposed on the cell surface (Fig. 50). Previous data suggested that this protein was a porin (section 6.1.1), and therefore, it would be expected to be on the cell surface. The predominant Mr 14,500 and 15,000 proteins of B1 were not detected.

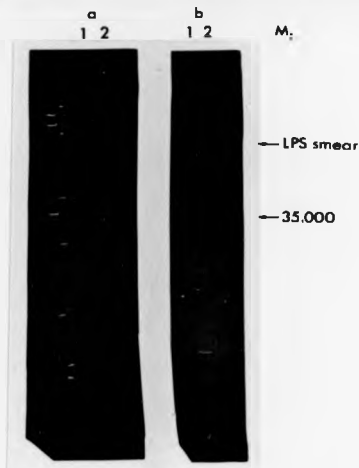
Figure 49

Inactivation of  $\phi$ BHG1 by cell wall fractions and  
cytoplasmic membrane from *Rp. blastica*

$10^5$  pfu  $\text{ml}^{-1}$   $\phi$ BHG1 were added to  $150 \mu\text{g ml}^{-1}$  of cell envelope fractions in 0.01 M Tris HCl, pH 6.8 at  $30^\circ\text{C}$ , samples were removed at varying times to titre active phage.  $\bullet$ ---- $\bullet$ , cell wall fraction B1;  $\blacktriangle$ ---- $\blacktriangle$ , cell wall fraction B2;  $\blacklozenge$ ---- $\blacklozenge$ , cytoplasmic membrane;  $10^5$  pfu  $\text{ml}^{-1}$   $\phi$ BHG1 in 0.01 M Tris-HCl, pH 6.8 showed no decrease in plaquing activity.







**Figure 20**

Protein immunoblots of cell wall fractions B1 and B2 from *Rp. blastica* and *Rp. blastica*<sup>r9</sup> with antisera raised against whole cells of *Rp. blastica* to determine exposed surface proteins

Antigens were detected by Western immunoblotting. 50 µg of cell wall fractions B1 and B2 were loaded onto a denaturing 10% (w/v) acrylamide gel. Detection of protein antigens was by the Amersham streptavidin-biotinylated peroxidase complex system. a) lane 1, cell wall fraction B2, *Rp. blastica*; lane 2, cell wall fraction B1, *Rp. blastica*. b) lane 1, cell wall fraction B2, *Rp. blastica*<sup>r9</sup>; lane 2, cell wall fraction B1, *Rp. blastica*<sup>r9</sup>.

This evidence suggested that those proteins, which were not found in *Rp. blastic*<sup>r9</sup>, were not exposed phage receptor proteins. The detection of only one cell wall protein was surprising considering the number of proteins constituting a cell wall. However, the LPS smear on the blots and the evidence that some kind of EPS material may be present in *Rp. blastic* suggested that many proteins located at the cell surface were obscured and therefore non-immunogenic. Had a distinct surface protein likely to be a phage receptor been identified then antisera would have been raised to this protein and further studies on phage adsorption and receptor localisation been carried out.

#### 6.6.6 Semi-denaturing gels

Alderman *et al.* (1979) devised a novel affinity technique coupled with semi-denaturing gel electrophoresis to identify the bacteriophage T6/colicin K receptor in *E. coli*. Phage were immobilised in a stacker gel and cell membranes, which had been semi-denatured to maintain biological activity, were run through the stacker into the resolving gel. Proteins involved with the adsorption of the phage became complexed with the phage and were therefore missing in the resolving gel. Cell wall fractions B1 and B2 from *Rp. blastic* when prepared and applied to a semi-denaturing gel gave only smearing in the stacker and top of the resolving gel. No discrete bands were observed suggesting a lack of solubilization and that the sample buffer could only be used with purified outer membranes.

## 6.7 Discussion

ØBHG1 was inactivated by cell wall fractions and by some component(s) of purified LPS from *Rp. blastica* which was absent in *Rp. blastica*<sup>r9</sup>. When LPS from *Rp. blastica* was sonicated the efficiency of inactivation of ØBHG1 was greatly reduced. Amino acid analysis suggested that contaminating proteins were present in the LPS and these were removed by the addition of pronase. After pronase treatment the LPS did not inactivate ØBHG1. These results suggested that ØBHG1 was inactivated by an LPS complex that required protein(s) association.

Evidence from pronase and heat pre-treatment of *Rp. blastica* suggested that no protein receptor was directly involved in phage adsorption. This, however, was not to say that protein(s) in some way may interact with LPS to form an 'active' complex as discussed above.

Cell wall fraction B1 contained two unique proteins Mr 14,500 and 15,000 which were present in only trace amounts in *Rp. blastica*<sup>r9</sup>. These would be obvious candidates for protein phage receptors, but the above results suggested there is no protein directly involved as a receptor, and only cell wall fraction B1 should be capable of inactivating ØBHG1 as fraction B2 contained only trace amounts of these two proteins. Data, however, showed that both cell wall fractions were equally capable of ØBHG1 inactivation, indicating that the receptor for ØBHG1 was not segregated into one cell wall fraction from disrupted cells. Protein immunoblotting also indicated that neither of these proteins was exposed to the cell surface. Only the heat modifiable protein of Mr 35,000 present in both cell wall fractions, and a LPS smear were detected.

#### 6.8 Conclusions

- a) LPS (WPH) isolated from *Rp. blastica* inactivated ØBHG1. Sonication and pronase treatment abolished this activity.
- b) LPS (WPH) from *Rp. blastica*<sup>r9</sup> did not inactivate ØBHG1.
- c) Heat and pronase pre-treatment of *Rp. blastica* cells had no effect on the rate of adsorption of ØBHG1.
- d) Cell wall fractions B1 and B2 from *Rp. blastica* inactivated ØBHG1 at the same rate. Cytoplasmic membrane was capable of limited phage inactivation.
- e) Only the Mr 35,000 heat modifiable protein present in both cell wall fractions appeared to be exposed on the cell surface.

**FINAL DISCUSSION**

## 7. Concluding Remarks

Very few phage have been isolated for members of the *Rhodospirillaceae* and only recently has interest in phage isolation and characterisation increased with the prospect of using phage as genetic tools in the study of phototrophic bacteria. Most phage of the *Rhodospirillaceae*, as with ØBHG1, have a virulent life cycle. ØBHG1 appears to be well adapted to the possible growth modes and environmental conditions experienced by its host *Rp. blastica* in its natural habitat, i.e. autotrophic water. For example, ØBHG1 does not appear to require cations for adsorption, such ions would be at minimal concentrations in the natural environment. Adsorption and the burst size of this phage are independent of the mode of growth of *Rp. blastica*, i.e. chemoheterotrophic or photoheterotrophic. This is important because of the ability of *Rp. blastica* to switch between the two modes of energy production, i.e. light or chemical oxidation, to survive (the relative contributions of each in the environment being difficult to ascertain). Unlike some coliphage, ØBHG1 replication appears to be closely related to the optimum growth rate of *Rp. blastica* at 30°C. It must be taken into consideration that such a temperature and laboratory culture conditions would almost certainly never be attained in the environment, making these studies a distortion of the 'real' interactions in the natural habitat.

ØBHG1 is unique amongst the *Rhodospirillaceae* phage because of its site of adsorption to *Rp. blastica*. This phage adsorbs specifically only to the old 'pole' of the dividing cell. This is in contrast to the 'lost' phage, Rpl, of *Rp. palustris* (Bosecker *et al.*, 1972b) which only attached to the actively growing pole and division plain. This specific adsorption site of ØBHG1 can be used as a marker for macromolecular

differentiation of the cell surface. Chemical analyses of cell wall fractions and lipopolysaccharide from *Rp. blastica* and a spontaneous phage resistant mutant *Rp. blastica*<sup>r9</sup> identified by a number of techniques, two major differences between the strains. First was the almost complete absence of the unusual neutral sugar 2-O-methyl-6-deoxyhexose (unique to *Rp. blastica*), and a reduction in the amount of galactose present in *Rp. blastica*<sup>r9</sup>. Secondly, two low molecular weight proteins of Mr 14,500 and 15,000 which appeared to be phosphorylated and present specifically in cell wall fraction B1 of *Rp. blastica*, were present in only trace amounts in *Rp. blastica*<sup>r9</sup>.

Accumulated evidence suggests that ØBHG1 is inactivated by an LPS/protein complex rather than a discrete protein or LPS receptor. A similar observation has been found in *E. coli* (Nikadio and Varra, 1985). The *E. coli* Omp F and Omp C porins act as receptors for phage Tula and phage Tuli and Mel respectively, but isolated porins do not inactivate these phage. However, when the porins were resuspended with LPS, phage inactivation was restored (Yu *et al.*, 1981). A somewhat different system exists in the case of the receptor for phage T4. LPS containing non-reducing terminal glucose can act as the receptor by itself, but LPS with different structures require the presence of Omp C protein to inactivate the phage (Yu and Mizushima, 1982).

It may be that an LPS/protein complex of *Rp. blastica* required for ØBHG1 adsorption is located at the 'old' pole of the cell. This would support the idea that the cell wall fraction B1 represents a distinct topographical region of the cell, possibly the polar or cap region. Results indicate that two proteins of molecular weights 14,500 and 15,000, absent in *Rp. blastica*<sup>r9</sup>, are specifically targeted to a defined



region of the cell. Recently Nathan *et al.* (1986) reported differential localisation of membrane chemotaxis receptor proteins in the *Caulobacter* predivisional cell, whilst Koyasu *et al.* (1983) reported the presence of different penicillin binding proteins in stalk preparations compared to total cell envelopes.

Electron micrographs suggest that the phage does not show lateral movement indicating that the receptor complex is 'fixed' only at the 'old' pole. As LPS is capable of lateral movement (Muhlradt and Menzel, 1974), the protein part of the complex must be the fixed component. Suitable candidates would be lipoproteins, i.e. proteins that are covalently bound to the peptidoglycan layer and therefore incapable of lateral movement. Data obtained does indicate that *Rp. blastic* possesses such proteins. Prime candidates would be the proteins of molecular weights 14,500 and 15,000 which appear to be phosphorylated, and located specifically in cell wall fraction B1. (This could be confirmed by radiolabelling with fatty acids). Assuming that cell wall fraction B1 represents the polar/cap region, where  $\phi$ BHG1 is adsorbed in whole cells, this fails to explain why both cell wall fractions B1 and B2 inactivate  $\phi$ BHG1 at an equal rate. One explanation may be that on breakage of the cells by French pressing, the integrity of the cell wall is disrupted resulting in non-specific inactivation of  $\phi$ BHG1 by both cell wall fractions. This would account for the high concentration of cell wall fractions required for the inactivation of  $\phi$ BHG1. An alternative interpretation is that breakage of the cells results in reduced fluidity of the membrane fragments. As a result LPS molecules located in the cell wall fraction B2 would become less mobile and therefore 'active', giving rise to the equal rates of inactivation of  $\phi$ BHG1 by both cell wall fractions. The poor non-specific adsorption of

ØBHC1 to *Rb. sphaeroides* 8253 suggests a degree of similarity between the steric arrangement and/or chemical composition of cell surface components when compared to *Rp. blastica*, supported by the evidence from neutral sugar analysis. Although there is no direct evidence for the presence of significant amounts of exopolysaccharide, some experimental data could be explained by the existence of it in trace amounts. Obviously, had there been significant amounts of EPS, its presence would have to have been taken into consideration in proposing the phage receptor model.

Although being the only phage which has been identified for *Rp. blastica*, it may not, and indeed is unlikely to be, the only phage capable of infecting it. Attempts were made to isolate further phage specific for *Rp. blastica* from its known autotrophic habitat using a virus concentrator and the isolation methods of Logan et al. (1981). Unfortunately, these attempts were unsuccessful.

To confirm the nature of the phage receptor for ØBHC1, isolation of the two proteins (molecular weights 14,500 and 15,000) enriched in cell wall fraction B1 of *Rp. blastica* would be required. The isolation procedure would be dependent on the nature of the proteins, i.e. lipoproteins bound to peptidoglycan or free proteins in the outer membrane (Rosenbusch, 1974; Flamman and Weckesser, 1984a), but problems are encountered with the complete removal of LPS. Extracted LPS would require digestion with pronase as a standard procedure before reconstitution with such isolated proteins. In an attempt to determine the importance of the terminal sugar residue in lipopolysaccharide, cells would be treated with sodium periodate, which is known to break the terminal glycosidic bond of an O-side chain, and the rate of

adsorption of ØBHG1 followed. The exact position of the cell wall fraction B1 in the whole cell could be determined by the use of immunogold electron microscopy.

From the data accumulated in this study it is obvious that ØBHG1 has some features in common with the few other phage characterised for members of the *Rhodospirillaceae*, but also has some novel properties. In particular, its specificity in adsorbing to only the 'old' pole of *Rp. blastica* cells should make it useful in future studies as a macromolecular marker of the cell surface to investigate differentiation events.

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